

ETIOLOGY AND EFFECTS OF CLOMIPHENE ON  
CYSTIC ENDOMETRIAL HYPERPLASIA IN THE MINIATURE PIG

1986

DUTTA

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## ABSTRACT

Title of Dissertation: Etiology and Effects of Clomiphene on Cystic Endometrial Hyperplasia in the Miniature Pig.

Chhanda Dutta, Doctor of Philosophy, 1986

Dissertation directed by: Prabir K. Chakraborty, Ph.D.  
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Cystic endometrial hyperplasia (CEH) of unknown etiology has been discovered in Swine Leukocyte Antigen (SLA) inbred miniature pigs. In humans endometrial hyperplasia is known most often to be associated with hyperestrogenism and thus it was hypothesized that CEH in SLA miniature pigs may also be due to hyperestrogenism. The present investigation was undertaken to perform a histological and biochemical characterization of CEH in miniature pigs. The primary objectives of this study were to: 1) document the gross morphological and histological changes of the endometrium associated with CEH, 2) determine whether differences exist in hormonal and/or steroid hormone receptor concentrations between CEH and non-CEH animals, 3) determine whether CEH can be induced by unopposed estrogen action and 4) examine the effect(s) of progesterone and clomiphene citrate on the progression of existing CEH condition.

The SLA miniature sows were assigned as CEH or non-CEH, based on the examination of the uterus by a midventral laparotomy. When necessary, animals were bilaterally ovariectomized during the laparotomy. Serum concentrations of estrone ( $E_1$ ), estradiol ( $E_2$ ), progester-

one (P), testosterone (T) and luteinizing hormone (LH) were measured by specific radioimmunoassays (RIA). Uterine tissue samples for histology and for quantitation of receptors were collected from sows at necropsy. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used for the qualitative analysis of proteins in cyst fluid, uterine flushings and serum samples. Endometrial estrogen and progesterone receptors were quantitated by radiolabelled ligand binding assay. Hemotoxylin and eosin staining of tissue was utilized for the histological analysis.

Examination of the gross morphology and histology revealed that the early stages of the development of CEH were characterized by the presence of small endometrial cysts (<10 mm in diameter) and an increase in the number of endometrial glands, but the architecture of the endometrium and myometrium were intact. In the advanced stages of CEH, the endometrial cysts were larger (10-50 mm in diameter) and numerous and this resulted in the complete disruption of the endometrium. However, the myometrium remained unaffected.

Electrophoretic analysis of cyst fluid revealed a protein (between 31K and 45K) not detected in uterine flushings nor in serum from either type of sow. Serum concentrations of  $E_1$ ,  $E_2$ , and T were not significantly different between CEH and non-CEH sows, during the synchronized estrous cycle. CEH sows had significantly lower ( $p < .01$ ) preovulatory LH surge and lower serum concentrations of progesterone during days 5 to 17 of the estrous cycle. The concentration of unoccupied cytosolic estrogen receptor in the endometria of CEH and non-CEH sows were not significantly different ( $p > .05$ ) during the luteal phase. The concentration of unoccupied nuclear estrogen receptors in the

endometrium of CEH sows was significantly lower ( $p < .05$ ) than that of non-CEH sows. Administration of unopposed 17- $\beta$ -estradiol to ovariectomized non-CEH sows resulted in excessive proliferation of the endometrial stroma and the stroma to gland ratio was increased. Cystic dilatation of the endometrial glands, however, was not evident.

Based on the classifications of endometrial hyperplasias in humans, CEH in SLA miniature pigs could be further classified as cystic glandular hyperplasia. The administration of progesterone or clomiphene citrate to intact CEH sows appeared to have little modifying effect on the progression of CEH. From the data obtained in the present investigation, it appears that CEH in SLA miniature pigs may be related to an imbalance between estrogen and progesterone concentrations during the estrous cycle. Short-term (90-days) progesterone or clomiphene citrate therapy appears to be ineffective against advanced CEH.

ETIOLOGY AND EFFECTS OF CLOMIPHENE ON

CYSTIC ENDOMETRIAL HYPERPLASIA  
IN THE MINIATURE PIG

by

Chhanda Dutta

Dissertation submitted to the Faculty of the Department of Pharmacology  
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#### DEDICATION

This dissertation is dedicated to my parents  
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## Chapter 1

### INTRODUCTION

#### Anatomy of the Uterus

In the female reproductive system, the shape and size of the uterus varies among species. In general, the uterus may be anatomically divided into the uterine horns, the body and the cervix. In humans, the uterus consists of two uterine (fallopian) tubes, the upper rounded portion of the uterus above the uterine tubes (fundus), the main portion of the uterus (body) and the lower, elongated portion called the cervix (Tortora, 1977). The paired broad ligaments support the uterus in the pelvic cavity, with the bladder located anteriorly and the rectum posteriorly.

In sows, the uterus is bicornuate (Hafez, 1980). Specifically, the uterine horns in the nonpregnant state will average between 40 to 65 cm in length. The body of the uterus is very small compared to that found in humans. The uterine horns are elongated in order to support multiple fetuses during gestation. Similar to humans, the uterus is attached to the pelvic cavity by the broad ligaments. In sows, however the uterus is positioned in the pelvic cavity such that the rectum is located dorsally and the bladder is located ventrally.

#### Histology of the Uterus

In humans, the uterus is a thick-walled, muscular organ which consists of three layers of tissue known as the serosa, myometrium and the endometrium (Tortora, 1977). The serosa, which is derived from the peritoneum, is the outermost layer. The middle layer (myometrium)

consists of smooth muscle fibers. The innermost layer of the uterus is called the endometrium. The endometrium consists of an epithelial layer which lines the uterine lumen and also of a glandular layer. The glandular layer can be further histologically classified into the stratum functionalis and the stratum basalis of the endometrium. The uterus of the sow is also composed of the serosal, myometrial and endometrial layers and the uterine histology in sows is very similar to that found in humans (Hafez, 1980).

#### Menstrual and Estrous Cycles

The primate reproductive cycle including humans is termed the menstrual cycle and is characterized by menstrual flow, which signals the start of a new cycle (Pritchard et al., 1980). The length of the menstrual cycle is usually 28 days and is defined as the time period from the initiation of one menstrual flow to the onset of the next menses. The first part of the menstrual cycle is termed the follicular phase and during this period there is an increase in the circulating concentration of estrogen, which is produced primarily by the growing follicle(s). This high estrogen concentration in the circulation induces proliferative changes in the endometrium and also triggers a preovulatory surge of luteinizing hormone, causing ovulation at midcycle (day 14). Following ovulation, luteinization of the ruptured follicle results in the formation of the corpus luteum which produces progesterone. The progesterone concentration in the circulation is maintained at a high level for 8-12 days and is responsible for the increased secretory activity in the endometrium. During late luteal phase there is an abrupt decline in the circulating concentration of progesterone resulting in the initiation of the next menstrual cycle.

The reproductive cycle in the sow which lacks menstruation, is known as the estrous cycle (Brinkley, 1981). The length of the estrous cycle is defined as the interval between the onset of one period of sexual receptivity (estrus) to the onset of the next estrus. Duration of estrus in the sow is usually 2-3 days. The length of the estrous cycle varies between 18 to 23 days. The first day that behavioral estrus is detected in the sow is designated as Day 0 of the estrus cycle. Ovulation in the sow occurs near the end of estrus and is characterized by the rupture of several follicles. Following ovulation, the progesterone level in the circulation rises and remains elevated for 16-17 days. Follicular growth is initiated 3 to 5 days following regression of the the corpora lutea, resulting in an increasing concentration of estrogens in the circulation. The abrupt fall in the circulating concentration of progesterone during the late luteal phase and the increasing concentration of estrogen signal the onset of the next behavioral estrus and ovulation.

#### Effects of Estrogen and Progesterone on Endometrial Morphology

Fluctuations in the circulating levels of estrogen and progesterone which occur during the menstrual and estrous cycles produce distinct morphological changes in uterine tissues of humans and more subtle changes in the sow. In general, estrogen promotes proliferative changes in uterine tissue (Pritchard et al., 1980). In humans, during the estrogenic phase of the menstrual cycle, estrogen primarily stimulates the growth of the endometrium. The increase in the thickness of the endometrium is accompanied by the lengthening of the tubular shaped endometrial glands. The composition of the endometrial surface epithelium also changes from that of cuboidal cells to columnar cells.

In the progestational phase, there is an increase in the secretory activity of the endometrium. Under the influence of progesterone endometrial glands accumulate uterine secretions and blood flow to the endometrium is increased. Progestational stimulation causes the endometrial glands to become coiled and tortuous. If fertilization occurs, the endometrium will thus be prepared for the implantation of the fertilized ovum. In the absence of implantation, the level of progesterone begins to fall towards the end of the menstrual cycle. The human endometrium undergoes necrotic changes and menstruation ensues. The subsequent rise in the level of estrogen will induce mitotic activity in uterine tissue and the menstrual cycle will be repeated.

In sows, during the follicular phase of the estrous cycle there is an increase in the vascularity and the length of the uterine glands in the endometrium (Hafez, 1980). In contrast to humans, the thickness of the endometrium increases during the early stages of the luteal phase of the estrous cycle by a synergistic effect of estrogen and progesterone. The uterine glands become coiled and there is increased secretory activity. If a pregnancy does not occur, the uterine glands regress in size and become inactive. There is also a regression in the thickness of the endometrium. A major difference between the menstrual and the estrous cycle is that there is no bleeding associated with the cyclic endometrial regression of the estrous cycle.

#### Distribution of Endometrial Steroid Hormone Receptors

The changes in endometrial morphology observed during the menstrual cycle result from the interaction of estrogen and progesterone with endometrial steroid hormone receptors. In a study by Tsibris et al. (1978) it was determined that in humans, the distribution of

endometrial estrogen and progesterone receptors differed along the length of the uterus. The concentration of cytoplasmic estrogen and progesterone receptors in endometrium was highest near the fundus of the uterus and lowest near the cervical area. In comparison to estrogen receptor concentration, the endometrium was found to contain a greater number of progesterone receptors. This gradient in the distribution of estrogen and progesterone receptors was maintained throughout the menstrual cycle. However, a difference was observed in the magnitude of the gradient between the estrogenic and progestational phases of the cycle.

These findings were further substantiated in another study by Tsibris et al. (1981). In this study the concentrations of both cytoplasmic and nuclear estrogen and progesterone receptors were measured in different sections of the uterus. The distribution of nuclear estrogen and progesterone receptors in the endometrium was found to be opposite that of cytoplasmic receptors. Mainly, in the endometrium the number of nuclear estrogen and progesterone receptors were found to be greater in the cervical area compared to the fundus of the uterus. Although the reason for the gradient in the steroid hormone receptor distribution remains to be delineated, the investigators suggested that the fundus with its higher ratio of cytoplasmic to nuclear estrogen and progesterone receptors, may represent a more proliferative tissue as compared to the cervical area.

## Cyclic Changes in the Concentration of Endometrial Steroid

### Hormone Receptors

In addition to the variation in the distribution of endometrial estrogen and progesterone receptors, the concentration of these receptors also fluctuate with the phases of the menstrual cycle (Muechler et al., 1975; Sanborn et al., 1978; Willson, 1983). A more recent investigation by Levy et al. (1980) documented the cyclic changes in estrogen and progesterone receptors in human endometrium during a normal menstrual cycle. An increase in total number of endometrial estrogen receptors was observed during the estrogenic or proliferative phase. However, when cytoplasmic and nuclear estrogen receptors were examined separately, it was found that the rise in the concentration of estrogen receptors was primarily due to an increase in the concentration of nuclear receptors. There was little change in the concentration of cytoplasmic estrogen receptors. Following ovulation, there was a sharp decline in the total number of estrogen receptors. A further decrease in estrogen receptor concentration occurred towards the end of the progestational or secretory phase of the cycle. Closer examination revealed that the postovulatory decline in estrogen receptor population was mainly due to a decline in the concentration of cytoplasmic receptors. The secondary fall in the concentration of estrogen receptors resulted from a decrease in the number of nuclear binding sites.

The preovulatory rise in progesterone receptor concentration was primarily due to a large increase in the number of cytoplasmic progesterone receptors. However, during the secretory phase there was a gradual fall in progesterone receptor concentration. Nuclear progesterone receptor concentration was found to be highest postovulation.

The increases in the concentration of estrogen and progesterone receptors during the estrogenic phase were also found to be associated with the significant preovulatory increase in the circulating level of estradiol. However, the changes in the population of estrogen and progesterone receptors in the progestational phase did not appear to be related to the circulating levels of estradiol or progesterone during the menstrual cycle. Similar findings were reported in studies by Robertson et al. (1971), Tseng et al. (1972) and Martin et al. (1979). Furthermore, no correlation was observed between the fluctuations in the population of endometrial estrogen and progesterone receptors and serum levels of luteinizing hormone, follicle stimulating hormone or prolactin (Spona et al., 1979). Disruption of the cyclic influence of estrogen and progesterone on the endometrium can lead to abnormal proliferation as observed with endometrial hyperplasia (Chamlain et al., 1970; Jones et al., 1982).

During the estrous cycle of sows, the concentration of cytoplasmic estrogen receptors in the endometrium begin to increase during the estrogenic phase and reaches a peak early in the progestational phase (Pack et al., 1978; Koziorowski et al., 1984). Thereafter, there is a gradual fall in the concentration of cytoplasmic estrogen receptors during the progestational phase until the next onset of estrus (Deaver et al., 1980; Rexroad et al., 1984). Peak concentrations of nuclear estrogen receptors are found during estrus and there is a gradual fall in the concentration of nuclear estrogen receptors during the progestational phase of the estrous cycle (Pack et al., 1978). The concentration of cytoplasmic progesterone receptors in the endometrium reaches a peak during estrus and is lowest or nondetectable during the

progestational phase (Koziorowski et al., 1984).

### Classification of Hyperplasias

The diagnosis of hyperplasia is mainly based upon histological examination of uterine tissue. A suggested classification of human endometrial hyperplasias based on histology includes: 1) cystic glandular hyperplasia, 2) adenomatoid hyperplasia, 3) glandular hyperplasia with architectural atypia and 4) glandular hyperplasia with cytologic atypia (Fox, 1984).

Unfortunately, these classifications are often misused and can also be misleading. For example in cystic glandular hyperplasia, there may be no glandular involvement in the hyperplastic condition. Adenomatoid hyperplasia is characterized by an excess of endometrial glands, the appearance of which is similar to that observed normally during the estrogenic phase of the reproductive cycle. Glandular hyperplasia with architectural atypia is distinguished by an abnormal appearance of the glands. The glandular epithelium protrudes into the surrounding stroma forming budlike projections. Glandular hyperplasia with cellular atypia is similar to the previous condition, except the cells of the glandular epithelium have nuclear and cytoplasmic abnormalities.

Since the terminology of and the morphological criteria for the classification of endometrial hyperplasia varies widely, an accurate diagnosis is still a major problem. Another difficulty lies in the fact that various forms of endometrial hyperplasia may exist simultaneously in the same endometrial tissue (Robbins et al., 1984). This suggests that different types of endometrial hyperplasia may result from either a common defect or perhaps one form of hyperplasia serves as a precursor to another form.

### Clinical Significance of Endometrial Hyperplasia

A possible association of human endometrial hyperplasia with endometrial carcinoma had been suggested by several early investigations (Novak et al., 1948; Hertig et al., 1949; Gusberg et al., 1954). More recently it has been proposed that specific types of endometrial hyperplasia may have a greater potential for progressing into carcinoma (Kistner, 1982; Bhagavan et al., 1984). For instance, atypical adenomatous hyperplasia appears to be a premalignant condition (Welch et al., 1977; Deligdisch et al., 1985). Furthermore, studies by Wagner et al. (1967) and Katayama et al. (1967) demonstrated that chromosome aberrations present in atypical adenomatous hyperplasia were similar to those observed in endometrial carcinoma. In a more recent investigation by Kurman et al. (1985) it was proposed that forms of endometrial hyperplasia possessing both cytologic and architectural atypia have a greater potential for malignancy than those forms with only cellular atypia. Despite such studies, a definite causal relationship between endometrial hyperplasia and carcinoma remains to be established.

This investigation was undertaken to characterize cystic endometrial hyperplasia (CEH) that was discovered in inbred miniature swine (Swine Leukocyte Antigen, SLA) developed by the National Cancer Institute for organ transplantation studies. This characterization of CEH includes both histological and biochemical studies. In humans endometrial hyperplasia is known most often to be associated with hyperestrogenism and thus it was hypothesized that CEH in SLA miniature pigs may also be due to hyperestrogenism. The primary objectives of this present investigation were to: 1) document the gross morphological and the histological changes associated with CEH, 2) examine the sex

steroid hormone profiles of CEH and non-CEH sows throughout the estrous cycle, 3) analyze the endometrial estrogen and progesterone receptor concentrations in CEH and non-CEH sows, 4) determine whether CEH may be induced by the administration of estrogen and 5) determine whether progesterone or clomiphene citrate therapy could alleviate this condition. In SLA miniature pigs, CEH appears to arise spontaneously in that no known exogenous chemical induction is required. The SLA miniature pig may serve as an important animal model in further investigations of endometrial hyperplasia.

## Chapter 2

### LITERATURE REVIEW

#### Etiology of Endometrial Hyperplasia

Under normal physiological conditions, estrogens promote cyclic proliferation of the endometrium. Abnormal growth of the endometrium, as seen in cystic endometrial hyperplasia (CEH) has been attributed to a prolonged, unopposed estrogenic influence on the endometrium (Jones et al. 1981). Endometrial hyperplasia may arise either from exogenous administration of estrogens or from pathological conditions. Specifically, any condition that results in an acyclic production of estrogen is likely to be associated with endometrial hyperplasia. Physiological disorders which have been observed with the occurrence of endometrial hyperplasia include obesity, diabetes mellitus and hypertension (Cramer et al., 1979). When hyperestrogenemia occurs with such conditions, it is mainly due to the increased capacity for estrogen production from androgens in the circulation (Hemsell et al., 1974; MacDonald et al., 1978). It remains to be determined whether the lowering of high blood pressure or the reduction of body weight in women with endometrial hyperplasia would result in the regression of this pathological condition.

When endometrial hyperplasia occurs during childbearing years, most frequently it is due to unopposed hyperestrogenemia associated with anovulatory menstrual cycles (Chamlain et al., 1970; Jones et al., 1982). The anovulatory menstrual cycle may be a result of the failure of one or several follicles to rupture and consequently these follicles continue to produce estrogen. Under such circumstances, there is excessive stimulation of the endometrium due to the acyclic production of estrogen.

Additional evidence for the role of estrogens in the etiology of endometrial hyperplasia was obtained from studies examining the incidence of this condition in postmenopausal women placed on estrogen replacement therapy. With the availability of oral estrogen preparations, estrogen replacement therapy for postmenopausal women has become widespread. Postmenopausal women are usually placed on estrogen therapy to provide relief of vasomotor flushes and vaginal irritation resulting from urogenital atrophy (Council Report, 1983). The administration of estrogens is also thought to prevent loss of bone mass and hip fractures (Lindsay et al., 1978; Christiansen et al., 1982). In the past, investigators had found a prevalence of endometrial hyperplasia in postmenopausal women given 1.25 mg or less of conjugated estrogens in a cyclic manner (Buchman et al., 1978; Rosenwaks et al., 1979). However a major fault with these studies was that pretreatment endometrial biopsies were not collected and thus the development of endometrial hyperplasia could not be specifically attributed to the estrogen treatment.

A recent investigation by Schiff et al. (1982) examined the effect on the endometrium of a cyclic estrogen regimen, as compared to a continuous estrogen regimen. Twenty-five symptomatic, postmenopausal women participated in the study. All of the women were in good health and had no contraindications for estrogen replacement therapy. The predominant symptom in both treatment groups was vasomotor flushes and the reproductive history in relation to parity was similar in both groups. Endometrial biopsies were obtained from the women at the beginning of the study in order to evaluate the condition of the endometrium. Of the 25 women, 12 were assigned to receive a cyclic estrogen regimen.

This consisted of three weeks of 0.625 mg Premarin (estrone sulfate and equilin), followed by one week of placebo. The remaining 13 women were placed on a continuous estrogen regimen of 0.625 mg Premarin daily. Both regimens of estrogen treatment were administered for one year. Endometrial biopsies were collected at the end of the study and it was found that the rate of endometrial hyperplasia was unacceptably high with both the cyclic and continuous regimens (48% and 44% respectively). This study demonstrated that even when the estrogenic stimulation of the endometrium was cyclic, endometrial hyperplasia developed probably because the estrogenic stimulation was unopposed.

In postmenopausal women, the small amounts of estrogen produced from extra-glandular aromatization of adrenal androgens can also lead to the development of endometrial hyperplasia. Experiments conducted by Sileri et al. (1974) demonstrated that the major source of estrogen in postmenopausal women came from the peripheral conversion of adrenal androstenedione to estrone. Furthermore, Schindler et al. (1972) found that the rate of peripheral conversion of androstenedione to estrone was approximately three times greater in postmenopausal women with endometrial hyperplasia in comparison with normal subjects.

Additionally, endometrial hyperplasia can occur under conditions where the level of estrogens in the circulation does not appear to be elevated. In a review by Gambrell et al. (1983) it was reported that the levels of sex hormone-binding globulin (SHBG) were reduced 60-80% in obese, postmenopausal women with and without endometrial cancer. Consequently, free estradiol levels in serum were increased two to three fold higher than normal. Other investigations have determined that SHBG concentration is inversely related to excess body weight

(Nisker et al., 1980). Thus when serum SHBG levels are reduced, there is more free estradiol available to act upon target tissues, such as the endometrium.

Recent experiments conducted by CioCCA et al. (1985) discovered a 24K protein which was secreted by cystic hyperplastic endometrium. Presence of this protein was most frequent in cystic hyperplasia as compared with other types of hyperplasia and carcinomas. In addition, it was suggested that the secretion of this protein was under estrogenic influence. Examination of normal endometria showed that the presence of the 24K protein was highest during the follicular phase, when estrogen levels are elevated. The significance of this protein in the development of endometrial hyperplasia in humans remains to be determined.

#### Endometrial Steroid Hormone Receptors and Endometrial Hyperplasia

Janne et al. (1979) quantitated cytoplasmic endometrial estrogen and progesterone receptors from patients with endometrial hyperplasia. In normal endometrium, estrogen receptor concentration was  $133 \pm 23$  fm/mg of protein and progesterone receptor concentration was  $842 \pm 152$  fm/mg of protein during the estrogenic phase of the menstrual cycle. In the progestational phase, the concentrations of estrogen and progesterone receptors were  $159 \pm 37$  and  $696 \pm 168$  fm/mg of protein, respectively. Hyperplastic endometrium was found to have a greater number of progesterone receptors than normal endometrium. The concentration of progesterone receptors in hyperplastic tissue was  $1683 \pm 403$  fm/mg of protein. The concentration of estrogen receptors ( $204 \pm 35$  fm/mg of protein) in hyperplastic endometrium was similar to that found in normal endometrium. In addition, there was no significant correla-

tion between the serum concentrations of estradiol, progesterone, luteinizing hormone and follicle stimulating hormone and the concentrations of endometrial estrogen and progesterone receptors in women with endometrial hyperplasia. Muechler et al. (1975) also reported that the concentration of cytoplasmic estrogen receptors in hyperplastic endometrium was within the range of estrogen receptor population of normal endometrium.

Other investigators examining the population of progesterone receptors in hyperplastic tissue have obtained results which contradict the findings of Janne and associates (1979). Rodriquez et al. (1979) found that the concentration of cytoplasmic progesterone receptors was highest towards the end of the estrogenic phase and near the beginning of the progestational phase. The concentration of progesterone receptors during the late proliferative phase and early secretory phase were  $640 \pm 355$  and  $824 \pm 169$  fm/mg of protein, respectively. The concentration of cytoplasmic progesterone receptors in hyperplastic endometrium (range between 58 to 547 fm/mg of protein) was found to be similar to that in normal endometrium. The data of this study were in agreement with the findings of a previous investigation by Haukkamaa et al. (1971). Furthermore MacLaughlin and coworkers (1976) showed the equilibrium constant of dissociation of progesterone receptors was similar in hyperplastic and normal endometria ( $3.3 \times 10^{-10}M$  and  $4.0 \times 10^{-10}M$ , respectively).

#### Therapeutic Regimens for Endometrial Hyperplasia

The type of treatment prescribed for endometrial hyperplasia is dependent upon the cause and severity of the condition. The therapy may consist either of curettage or the administration of progestational

compounds, when endometrial hyperplasia develops in women of child-bearing age (Jones et al., 1982). Specifically, if the condition is noninvasive, then the hyperplastic tissue is removed by curettage. When ovarian dysfunction is the cause, progestational compounds are administered.

A more vigorous therapy is given to postmenopausal patients with endometrial hyperplasia. One of the first studies that examined the histological effect of progestogen on hyperplastic endometrium was conducted by Kistner (1959). It was observed that menopausal and postmenopausal women with severe endometrial hyperplasia when given progestins such as mestranol, norethynodrel or 17- $\alpha$ -hydroxyprogesterone caproate underwent a regression of the hyperplastic condition. Similar findings were obtained by Eichner et al. (1971). In their investigation, women between the ages of 36 to 60 years with endometrial hyperplasia received either 80 mg megestrol daily for nine weeks or 80 mg medroxyprogesterone for a period of six weeks. This treatment resulted in complete remission of the condition. However the duration of remission varied in the subjects, the longest being four years.

Another study determined that continuous progesterone therapy was effective in preventing the recurrence of endometrial hyperplasia in postmenopausal women (Gal et al., 1983). Fifty-two postmenopausal subjects with various degrees of endometrial hyperplasia at the beginning of the study received 40 mg of megestrol acetate daily for an average period of 42 months. Curettage was performed every six months to evaluate the status of endometrium. There were mild side effects from therapy such as nausea, however, there were no serious complications. At the conclusion of the study, curettage was performed

and it was found that 90% of the subjects were in complete remission of endometrial hyperplasia. Thus it was proposed that a continuous regimen of megestrol acetate was a safe and effective alternative to hysterectomies for treatment of endometrial hyperplasia in postmenopausal women.

A recent investigation by Wentz (1985) examined the effectiveness of megesterol acetate in the treatment of persistent endometrial hyperplasia. Women participating in this study were either of child-bearing age or were postmenopausal. All subjects received 20 mg megesterol acetate four times daily, for a period of eight weeks. Endometrial specimens were collected two months after the withdrawal of progestin treatment and at specified intervals thereafter for a period between one to four years. Endometrial morphology was found to be normal during this follow-up period. Therefore it was concluded that megesterol acetate is effective against persistent endometrial hyperplasia in both young and postmenopausal women.

#### Sequential Estrogen/Progestin Therapy and Endometrial Hyperplasia

Since cyclic unopposed estrogen therapy can result in the development of endometrial hyperplasia and progestins produce a regression of this condition, Campbell et al. (1978) hypothesized that the addition of a progestin to estrogen therapy may offer protection against the development of endometrial hyperplasia. The subjects had endometrial hyperplasia either from exogenous estrogens or the condition had arisen spontaneously. The two types of hormonal therapy evaluated in this investigation were an unopposed cyclic estrogen regimen and a sequential estrogen/progestin regimen.

The cyclic estrogen therapy consisted of either a high or low dose of estrogens. High dose estrogen therapy was composed of 1.25 mg/day of conjugated equine estrogen preparation or 3.0 mg/day of piperazine estrone sulfate or 2.0 mg/day of estradiol valerate. These estrogenic compounds were used at half their respective doses, for the low dose estrogen therapy. Women assigned to the sequential high dose estrogen/progestin therapy received estrogens (same doses as used in cyclic high dose estrogen regimen) and 5.0 mg of a progestin (norethisterone and medroxyprogesterone acetate) daily. The doses of estrogens and progestins were halved for the sequential low dose estrogen/progestin therapy. Further classifications of the sequential estrogen/progestin therapy included a continuous sequential regimen (daily uninterrupted administration of an estrogen with the progestin added for one week of each month) and a cyclic-sequential regimen (daily administration of an estrogen for three weeks followed by one week of progestin only, each month). Duration of all treatments was four years. The condition of the endometrium during the treatment period was monitored by curettage.

Results of the study showed that in the group of women receiving cyclical estrogen therapy, the incidence of endometrial hyperplasia was unacceptably high with both high and low dose regimens (33% and 18%, respectively). In contrast, the occurrence of endometrial hyperplasia was infrequent in subjects receiving either continuous or cyclical, sequential estrogen/progestin therapy. More importantly, there was no evidence of endometrial hyperplasia in women placed on sequential low dose estrogen/progestin therapy. The investigators concluded that a sequential estrogen/progestin regimen is a suitable form of therapy for

postmenopausal women, since it was effective against endometrial hyperplasia whether it had arisen spontaneously or was induced by unopposed estrogen replacement therapy.

Despite the successful use of progestational compounds in the prevention and treatment of endometrial hyperplasia, there are some associated disadvantages. Primarily, progestins are only a palliative cure for endometrial hyperplasia. Under the influence of progesterone, a hyperplastic endometrium regresses to its normal state. However, once the therapy is stopped, there is a relapse of endometrial hyperplasia (Chamlain et al., 1970). This is especially frustrating to young women with endometrial hyperplasia who wish to have children and in order to conceive they cannot be maintained on progestin treatment. Consequently, these women have a small chance of conceiving whether they are maintained or withdrawn from progestin therapy.

Addition of progestogens to estrogen replacement therapy may also have an adverse effect on serum lipoproteins. Increased blood levels of very low density lipoprotein (VLDL) and/or low density lipoprotein (LDL) are related to an increased risk of cardiovascular disease, while an increased level of high density lipoprotein (HDL) appear to have a protective effect against the development of heart disease (Guyton, 1981). Krauss et al. (1978) reported that menopausal women on estrogen therapy had an increased level of HDL, in comparison to untreated menopausal women. However, when a progestin such as norethinodrone acetate was administered in combination with estrogens, the circulating level of HDL decreased. Although progestins produce a regression of endometrial hyperplasia, these compounds may also modify the beneficial effects of estrogen on the cardiovascular system as

demonstrated by this study. Subsequently, the relative risks and benefits of administering progestins with long-term estrogen replacement therapy remains to be clarified.

#### Proposed Mechanism(s) of Progestin Action

The ability of progesterone to modify or antagonize the effects of estrogen on the uterus has been examined by several investigators (Hsueh et al., 1975; 1976; Bhakoo et al., 1977; Isomaa et al., 1979; Martin et al., 1979). Gurpide et al. (1976) found that progesterone administration reduced endometrial estradiol receptor concentration. In this study, estradiol receptor concentration was measured in endometrial tissue obtained from normal, menstruating women and from postmenopausal women with hyperplasia. The results showed that in a normal menstrual cycle, nuclear estradiol receptor concentration was high (3.1 pmol/mg DNA) during the estrogenic phase, whereas during the progestational phase, there were fewer (0.6 pmol/mg DNA) nuclear estradiol receptors in the endometrium. In order to show that this decline in the population of nuclear estradiol receptors was a result of progestational stimulation, some of the women were given Provera (synthetic progestogen) during the proliferative phase of the menstrual cycle. The administration of Provera during the luteal phase also caused a reduction in the population of nuclear estradiol receptors (1.5 pmol/mg DNA). The nuclear estradiol receptor concentration of hyperplastic endometrium was similar to the concentration found in normal endometrium during the estrogenic phase. Thus it was proposed that one of the ways in which progesterone modifies the hyperplastic state is by reducing the numbers of receptors available for estradiol binding. These investigators also measured the activity of estradiol dehydrogen-

ase in the endometrium during the proliferative and secretory phases of the menstrual cycle. Estradiol dehydrogenase is involved in the metabolism of estradiol to estrone. There was approximately a fourteen-fold increase in enzymatic activity during the secretory phase, in comparison to the proliferative phase. In addition, Provera treatment during the proliferative phase resulted in an increase in estradiol dehydrogenase activity. In vitro experiments revealed that the increase in enzymatic activity was due to induction of estradiol dehydrogenase. Furthermore, this induction could be blocked by actinomycin D, puromycin, or cyclohexamide.

The molecular mechanism by which progestins produce a regression of endometrial hyperplasia appears to be mediated through a reduction in the number of hormone-receptor complexes. Due to the accelerated endometrial metabolism of estradiol to estrone there is a lower amount of estradiol to complex with receptors. Additionally, the reduced number of nuclear receptors probably diminishes any estrogen stimulated cellular synthesis.

#### Effects of Antiestrogenic Compounds on the Uterus

Originally antiestrogenic compounds such as clomiphene and tamoxifen were developed for use as antifertility drugs. However, many clinical investigations have focused on the use of clomiphene to induce ovulation (Greenblatt et al., 1961; 1962; Garcia et al., 1977; Drake et al., 1978; Huppert et al., 1979). A forerunner compound of clomiphene, known as MER-25, was reported to induce ovulation in women with Stein-Leventhal syndrome and this was accompanied by a simultaneous regression of endometrial hyperplasia also present in these women (Kistner et al., 1961). A review of current clinical literature reveals that very few

studies have examined the use of antiestrogenic compounds in the treatment of endometrial hyperplasia, be it in young or in postmenopausal women.

In addition to their antiestrogenic properties, compounds such as clomiphene and tamoxifen in many species have inherent weak estrogenic activity. Gulino et al. (1984) evaluated the morphological effects of estradiol and tamoxifen on uterine tissue of immature guinea pigs (27 day old). Estradiol administration (30 ng/day) for six days resulted in an overall increase in the size of the uterine horns, as well in the uterine DNA content. Histological examination revealed that there was an increase in the number and the height of the endometrial epithelial cells upon estradiol treatment. A hypertrophic effect on the myometrial layer was observed following estradiol administration and the development of uterine glands was also enhanced. In contrast to estradiol, tamoxifen (0.6 µg/g body weight) possessed only weak estrogenic effects on the immature uterus. Uterine wet weight gain and the increase in uterine DNA content was less pronounced than that induced by estradiol. The effect of tamoxifen on endometrial epithelial cell height and on the number of uterine glands was minimal in comparison to that of estradiol. Furthermore, both the number of epithelial cells and the thickness of the myometrial layer was unaffected by tamoxifen.

An earlier study by Black et al. (1980) had examined the effect of tamoxifen on uteri of immature rats and mice, as well as adult, ovariectomized mice. In immature rats, the uterine growth observed with tamoxifen (1 mg/day) was less than that seen with estradiol (10 µg/day) following a treatment period of four days. When different doses (3 to 1000 µg/day) of tamoxifen were administered with 0.1, 1 or

10 µg/day of estradiol, there was a dose-dependent inhibition of the uterotrophic response to estradiol. The lowest dose of tamoxifen had little effect on the uterotrophic response when used in combination with 0.1 µg of estradiol. Similar results were obtained with immature mice. When tamoxifen (10 µg) was administered to adult, ovariectomized mice, the uterotrophic response was similar to that of estradiol (1 µg). In addition, tamoxifen was not able to inhibit the uterotrophic effect of estradiol, when given together. The cause for the developmental alteration in the uterine response to tamoxifen remains to be determined.

Poteat et al. (1975) examined the estrogenic and antiestrogenic properties of clomiphene. In this study, ovariectomized rats received either estradiol (1 µg), or clomiphene (50 µg), or both estradiol and clomiphene, daily for three days. The increase in uterine size was indistinguishable between the estradiol only and clomiphene only treatment groups. Furthermore, combination of estradiol and clomiphene resulted in hypertrophy of the endometrial epithelium. The degree of estrogenic activity of clomiphene was determined by the amount of uterine glycogen synthesis. Clomiphene only or in combination with estradiol resulted in an increased glycogen content of the endometrium. However, estradiol treatment alone did not produce an increase in glycogen synthesis in the endometrium although it did do so in the myometrium. Although clomiphene also caused glycogen accumulation in the myometrium, it was less than that observed with estradiol used alone. When both estradiol and clomiphene were given, clomiphene was able to suppress the amount of myometrial glycogenesis stimulated by estradiol. These investigators concluded that clomiphene had a stronger

effect on endometrial epithelial cells than myometrial cells. Furthermore, the antiestrogenic effect of clomiphene was restricted to the myometrium.

#### Proposed Mechanism(s) of Action of Antiestrogens

The molecular mechanism(s) by which antiestrogens exert their agonistic and/or antagonistic properties on various estrogen target tissues is not clear. The antagonistic effects have been suggested to be attributed to nuclear accumulation of antiestrogen-estrogen receptor complexes and the subsequent depletion of cytoplasmic estrogen receptors (Clark et al., 1973; 1974; Katzenellenbogen et al., 1975; 1977). If the cytoplasmic pool of estrogen receptors is depleted, it would preclude any further estrogenic stimulation of the tissue. Although this theory is generally accepted, it does not appear to be complete. It is not known if and how antiestrogen-estrogen receptor complexes are processed once they are in the nucleus. Furthermore, it is not known whether nuclear processing is necessary for replenishment of the cytoplasmic estrogen receptor pool. A prolonged nuclear retention of the antiestrogen-estrogen receptor complex has been proposed to explain the agonistic properties of antiestrogens (Clark et al., 1973; 1976). If one assumes that nuclear processing of estrogen-estrogen receptor complex occur following interaction with specific nuclear sites and for some unexplained reason the antiestrogen-estrogen receptor complex is not susceptible to this processing, it can then remain in the nucleus for a longer time. The reduced uterotrophic effect of antiestrogens may thus be due to a lower, intrinsic biological activity of the antiestrogen-estrogen receptor complex, in comparison to that of estrogen-estrogen receptor complex.

### Animal Models for Endometrial Hyperplasia

At the present time there does not appear to be a suitable animal model for spontaneously-arising endometrial hyperplasia in women. Most studies designed to investigate this condition have induced endometrial hyperplasia in rodents utilizing both mice and rats. Kimura (1986) used 3-methylcholanthrene (MCA) to induce endometrial hyperplasia in mice. In this study, laparotomy was performed in mice in order to place a cotton string coated with MCA and beeswax into the uterine cavity. Endometrial hyperplasia was evident by the fourth week of exposure to MCA and after 20 weeks, adenocarcinoma was present. Total of 2.5 mg of progesterone over a 60 hr period was administered subcutaneously on the 4th week after MCA implantation and mitotic activity was examined. Mitotic activity in the endometrium was measured by  $^3\text{H}$ -thymidine incorporation. The  $^3\text{H}$ -thymidine uptake was reduced in progesterone treated mice in comparison to untreated mice. This study indicated that the main effect of progesterone on endometrial hyperplasia was to arrest mitotic activity.

Tang et al. (1984) used Fischer rats as a model for endometrial hyperplasia. In this experiment estradiol pellets designed to provide serum levels of 110-260 pg/ml were implanted subcutaneously into 12 mo old, ovariectomized Fischer rats. These animals were sacrificed upon evidence of endometrial hyperplasia (approximately nine mo following implantation of pellets). Monolayer cultures of endometrial cells were established and used to examine the effect of progesterone (10mM) on cell proliferation. Mitotic activity was determined by  $^3\text{H}$ -thymidine incorporation. In support of Kimura's findings (1978), progesterone exposure led to mitotic arrest of endometrial cells from hyperplastic endometrium.

### Reports of Endometrial Hyperplasia in Other Species

Endometrial hyperplasia has been observed in a variety of species (Jones et al., 1983). This condition has been frequently reported to occur in dogs. In dogs, endometrial hyperplasia is thought to result from a prolonged stimulation of the endometrium by progesterone. The role of progesterone in the etiology of endometrial hyperplasia is further corroborated by the observation that endometrial hyperplasia cannot be induced in dogs by the administration of exogenous estrogens, including stilbesterol. Although cystic endometrial hyperplasia also occurs in horses and cats, the etiology of this condition in these animals is unknown. In cattle and sheep, endometrial hyperplasia is proposed to be due to hyperestrogenism. Additionally, there have been infrequent reports of cystic endometrial hyperplasia in domestic pigs (Thain, 1965; Singh et al., 1975). In both these reports, the endometrium from sows were observed to contain fluid-filled cysts of various sizes. However the hormonal factors responsible for the development of endometrial hyperplasia in these sows were not known. The occurrence of endometrial abnormalities in the SLA miniature pigs was first reported by Diehl et al. (1984). The endometrium of affected sows was found to contain large cysts, which were filled with either a clear or yellowish fluid. Upon pathological examination of endometrial specimens, this condition was diagnosed as cystic endometrial hyperplasia (CEH).

### Development of Swine Leukocyte Antigen (SLA) Inbred Miniature Swine

The Immunology Branch of the National Cancer Institute initiated a program to develop a strain of miniature pigs for the purpose of organ transplantation studies. The boar and sow which were mated to begin this strain came from two, independently established herds of miniature pigs (Sachs et al., 1976). The initial boar was obtained from Vita Vet Laboratories and the original sow was obtained from Hormel Institute. These pigs are classified as miniature because they attain an adult weight of 113 kg, whereas adult standard-sized pigs can weigh up to 363 kg. These pigs have been selectively inbred over several generations to produce animals which are homozygous at loci of the miniature swine leukocyte antigen complex (MSLA). The MSLA of miniature swine is comparable to the major histocompatibility complex (MHC) of humans. The degree of homozygosity at the MSLA is important in determining the survival of an organ transplant.

### Reproductive Characteristics of SLA Miniature Swine

Sexual maturity of miniature sows is attained at about five mo of age and standard-sized sows are sexually mature at five to seven mo of age (Haring et al., 1966). It has been observed that the litter size of miniature pigs was between four to six offspring/female, at weaning. This is approximately one-half the litter size of standard-sized pigs. An examination of the ovarian morphology of these pigs revealed that the reduced litter size was directly attributable to a fewer number of ovulations as compared to standard-sized pigs (Howard et al., 1982). The number of ovulations were determined by the number of corpora lutea (CL) present on the ovaries. In SLA miniature pigs, the average number of ovulations was nine CL/female; in comparison,

the number of ovulations in standard-sized pigs is usually 14 or more CL/female. The SLA miniature pigs are however, similar to standard-sized pigs with respect to the duration of estrus (two to three days) and the length of the estrous cycle (20-21 days). The temporal changes in the circulating levels of sex steroid hormones during the estrous cycle were found to be similar between SLA miniature sows and standard-sized pigs (Howard et al., 1983).

Since the reduced litter size in SLA miniature pigs was found to be due to the low ovulation rate, Diehl et al. (1984) conducted a study to determine whether superovulation and embryo transfer techniques could be utilized to increase the number of SLA miniature pigs. The SLA miniature sows were successfully superovulated and then mated. The presence of cystic endometrial hyperplasia was first detected during the embryo collection procedure. The uterine horns of some of the SLA miniature sows could not be easily flushed and thus embryo collection was difficult. The uterine lumen was found to contain endometrial cysts of varying size. Since the presence of cystic endometrial hyperplasia apparently interferes with normal reproductive function, it is important to further characterize this condition.

## Chapter 3

### MATERIALS AND METHODS

#### Description of General Research Techniques:

##### Maintenance of Animals During Experiments

In all the experiments performed, the SLA miniature pigs were housed in indoor/outdoor pens (approximately 5.6 m<sup>2</sup>/animal) at the National Institutes of Health Animal Center (Poolesville, MD). Sows were fed Purina Complete Sow Chow (1.0 - 1.5 kg/day, Ralston Purina Co., St. Louis, MO), twice daily. Water was available ad libitum.

##### Collection of Blood Samples

Sterile syringes and 19-20 gauge needles (Monoject, St. Louis, MO) were used to collect blood samples (8 ml) either from a leg vein or the jugular vein. Immediately after collection, blood samples were transferred into plain vacutainers (Becton-Dickinson, Rutherford, NJ). The samples were allowed to stand 1 hr at room temperature prior to centrifugation (1000 x g for 15 min) and the serum was removed. Serum samples were stored at -20°C until analyzed for hormone concentrations.

##### Collection of Uterine Flushings and Endometrial Cyst Fluid

Uterine flushings were obtained from non-CEH animals by injecting approximately 50 ml of PBS (0.14M NaCl, 0.01M NaPO<sub>4</sub>, with 1:10,000 merthiolate, pH 7.4) into each of the uterine horns. The uterine horns were tipped back and forth in order to thoroughly flush the uterine lumen. The ovarian end of each horn was cut and the fluid was drained into plastic centrifuge tubes. Endometrial cysts (>10 mm diameter) were aspirated with sterile syringes and 23 gauge needles to collect

cyst fluid. Samples of uterine flushings and endometrial cyst fluid were stored at  $-20^{\circ}\text{C}$ , until further analysis.

#### Preparation of Pituitaries for RIA

The pituitary glands were prepared as described by Chakraborty et al. (1973). The pituitary glands were removed from CEH and non-CEH sows immediately following sacrifice and quickly frozen in liquid nitrogen. Samples were stored at  $-80^{\circ}\text{C}$ , until assayed. Distilled water (2 ml) was added to pituitary glands and then homogenized in a ground glass homogenizer. Based on the weight of the pituitary glands, an appropriate amount of distilled water was added to the homogenate, to obtain a concentration of 10 mg/ml. Following centrifugation ( $250 \times g$  for 15 min), the supernatant was collected. The supernatant was further diluted with PBS buffer to a final concentration of 10  $\mu\text{g}/\text{ml}$  prior to addition to the assay. Concentration of LH in pituitary glands was expressed as  $\mu\text{g}/\text{mg}$  tissue.

#### Collection and Staining of Histological Specimens

Tissue specimens were collected from CEH and non-CEH sows at necropsy. The uterus was quickly removed and split lengthwise in order to expose the uterine lumen. Small pieces of uterine tissue (approximately 7 mm x 5 mm in size), were placed into tissue cassettes containing embedding compound (O.C.T. Compound, Miles Laboratories, Naperville, IL) and sprayed with Histo Freeze (Fisher Scientific Co., Pittsburgh, PA). The tissue specimens were stored at  $-80^{\circ}\text{C}$ , and cross-sections were prepared for histological examination. The uterine tissue specimens were sectioned (10 microns in thickness) with a cryostat. Hemotoxylin and eosin staining of frozen tissue sections was performed as recommended

by Bancroft et al. (1984).

#### Collection of Endometrial Tissue for Receptor Assays

Endometrial tissue specimens from CEH and non-CEH sows were also collected immediately after sacrifice. Small pieces of endometrial tissue were dissected from the midsection of both uterine horns and immediately frozen in liquid nitrogen. Samples were then stored at  $-80^{\circ}\text{C}$  until processed for the quantitation of estrogen and progesterone receptors.

#### Surgical Procedure

##### Anesthesia:

Atropine sulfate (0.07 mg/kg i.m., Atropine 15, A.J. Buck, Cockeysville, MD) was used as the preanesthetic medicant. Anesthesia was induced with an intramuscular injection of ketamine hydrochloride (20 mg/kg, Vetalar, Parke-Davis, Morris Plains, NJ) or with an intravenous (external ear vein) administration of thiamylal sodium (17.6 mg/kg, Bio-Tal, Boehringer Ingelheim Animal Health Inc., Indianapolis, IN). Following endotracheal intubation, the animals were placed under gas anesthesia. The endotracheal tube was connected to a closed circuit anesthetic system. The concentration of anesthetic used following intubation was 5% halothane and was reduced to 2-4% halothane after five min to maintain the sows in a surgical plane of anesthesia. Oxygen was delivered at a flow rate of 11.0 ml/kg/min.

##### Laparotomy and Ovariectomy:

Once a surgical plane of anesthesia was reached, the uterus was exteriorized via a midline laparotomy. Incisions between 12-14 cm in length were made through the skin (beginning at a point 6 cm posterior

to the umbilicus), muscular and peritoneal layers. The ovaries were located by following the length of the uterine horns in the pelvic cavity. Blood supply to the ovaries was blocked by using a purse string suturing technique around the base of the ovaries. Next the end of the oviduct near the fimbria was clamped and the ovaries were removed with a scalpel. Following the ovariectomy procedure, the peritoneal and muscular layers were sutured with chromic gut. The subcutaneous and the skin incisions were sutured with Dexon. An antibiotic (nitrofurazone, Nitro-Fur Puffer, Life Sciences Products) was topically applied to the incision site following surgery. A long-acting (2-3 days) preparation of oxytetracycline (18 mg/kg im, Liquamycin LA 200, Pfizer Inc.) was used as the postoperative antibiotic.

#### Electrophoresis

A vertical slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) was used for sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) of cyst fluid, uterine flushings and serum from CEH and non-CEH animals by the method of Laemmli (1970) with some modifications (Gordon, 1975; Hames, 1984). The dimensions of the gels were 14 x 16 cm and 1.5 mm in thickness. High and low molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were run with each gel. The composition of the high molecular weight standards and their respective molecular weights were as follows: myosin 200,000;  $\beta$ -galactosidase 116,250; phosphorylase B 92,500; bovine serum albumin 66,200 and ovalbumin 45,000. In addition, the low molecular weight standards included: carbonic anhydrase 31,000; soybean trypsin inhibitor 21,500 and lysozyme 14,400. Preparation of buffers and reagents for SDS electrophoresis are described in Appendix I.

#### Preparation of Resolving Gel:

Gradient polyacrylamide gels were used as resolving gels. Stock reagents used in gels included a solution "A", [30% acrylamide, 0.8% Bis acrylamide (Bio-Rad Laboratories, Richmond, CA)], solution "B" (0.375M Tris, 0.1% SDS, 9.0M urea, pH 9.0) and 50% sucrose solution (dissolved in solution "B"). Gases were removed from monomer solutions under vacuum, prior to pouring gels. Gradients were poured from the bottom to the top of the resolving gel, starting with a monomer solution of 15% T (50% solution "A", 21% solution "B", 29% sucrose solution) which was gradually diluted by 8% T (27% solution "A", 73% solution "B") with a gradient maker (Hoefer Scientific Instruments). Polymerization was initiated by the addition of ammonium persulfate (0.1 mg/ml, Bio-Rad Laboratories) and tetramethylethylenediamine (TEMED, Bio-Rad Laboratories) to each monomer solution just before pouring of the resolving gel. The gels were allowed 30-40 min for polymerization.

#### Preparation of Stacking Gel:

Stock reagents for this gel were the same as those for the resolving gel, except solution "C" (0.12M Tris, 0.1% SDS, pH 6.8) was substituted for solution "B". The stacking gel (14% solution "A", 86% solution "C") was quickly poured over the resolving gel, after the addition of ammonium persulfate and TEMED. A teflon comb (25 mm teeth, Hoefer Scientific Instruments) was then placed into the stacking gel. After polymerization (5-10 min) the comb was removed and the wells were filled with electrode buffer (0.02M Tris, 0.192M glycine, 0.1% SDS, pH 8.3).

#### Preparation of Samples:

Aliquots of uterine flushings, cyst fluid and serum were diluted 1:4 with sample buffer (5% SDS, 10% glycerol, 0.005% bromophenol blue, 2% mercaptoethanol) and then placed in boiling water for 4 min. After transferring the glass plates containing the gels into the electrophoretic cell, the samples (0.05 ml) were applied to the wells of the stacking gel. The gels were allowed to run overnight at constant voltage (70 V, Model 500 Power Supply, Bio-Rad Laboratories).

#### Staining and Drying of Gels:

Gels were placed in fixative (33% methanol) for a minimum of 2 hr prior to staining. The gels were stained with Coomassie Blue solution for 5 min. Excess stain was removed by soaking the gels in destainer (7% acetic acid). The gels were treated with a pre-drying solution (30% methanol, 3% glycerol, 3% stock acrylamide solution) and then placed into a dryer (1-2 hr, Dryjel Jr., Hoefer Scientific Instruments).

#### Radioimmunoassays (RIA)

##### Steroid Extraction Procedure:

Steroids were extracted from samples as recommended by Wildt et al. (1979). The amount of steroid recovered (% recovery) from ether extraction was determined by the addition of 0.1 ml of the appropriate tritiated hormone (1000-1200 cpm/0.1 ml) to samples prior to the addition of diethyl ether. Diethyl ether (5 ml) was added to aliquots (1 ml) of serum and of cyst fluid samples and vortexed (30 sec). These samples were then placed into liquid nitrogen to rapidly freeze the aqueous layer. The remaining organic layer was transferred into glass scintillation vials (Wheaton Scientific, Millville, NJ) and evaporated

to dryness (Multivap Organomation Associates Inc., Shrewsbury, MA). The samples were reconstituted with ethanol (1 ml) and allowed to stand (1 hr at room temperature) prior to determination of steroid recovery. Aliquots (0.1 ml) of the extracted samples were counted in a beta counter (Mark III, Model 6882, Tracor Analytic, Elk Grove Village, IL). Each sample was counted for 5 min. Percent recovery was calculated as the percentage of total counts remaining in the extracted samples.

Steroid Hormone Assays:

Quantitation of estrone ( $E_1$ ) was performed by the previously validated radioimmunoassay method of Guthrie et al. (1979) and estradiol ( $E_2$ ) by the method of Korenman et al. (1974), respectively. Preparation of assay reagents and buffers are described in Appendix II. Standard solutions of  $E_1$  or  $E_2$  were used with each assay at concentrations between 3 to 200 pg/assay tube to construct the standard curve. Thus the minimum amount of  $E_1$  or  $E_2$  detectable by these assays was 3 pg/assay tube. The  $E_1$  antibody (Ab #WR11 BARC #4) was used at a dilution of 1:100,000 and the  $E_2$  antibody (#244) was used at a 1:40,000 dilution. The antibodies and the tritiated hormones were diluted with 0.1% PBS-G. The radiolabelled hormones used were [2,4,6,7<sup>3</sup>H]estrone (specific activity 85-110 Ci/mMol, Amersham, Arlington Heights, IL) and [2,4,6,7<sup>3</sup>H]estradiol (specific activity 80-110 Ci/mMol, Amersham, Arlington Heights, IL).

Extracted samples were aliquoted (0.25 ml) in duplicate and dried with air in an evaporator (Multivap Organomation Associates Inc., South Berlin, MA) for 30 min. Tritiated estrone or estradiol (5  $\mu$ Ci/0.1 ml) and the appropriate antibody were added to the samples (final incubation volume was 0.2 ml), vortexed and then incubated (24

hr at 4°C). Following incubation, 0.5 ml of dextran-charcoal suspension was added to the samples. The mixture was allowed to incubate for an additional 10 min prior to centrifugation (1000 g for 10 min, Sorvall, Wilmington, DE). The supernatant was poured into biovials and 2.5 ml of scintillation cocktail (Hydrofluor, National Diagnostics, Sommerville, NJ) was added and each sample was counted for 1 min in a beta counter (Mark III, Model 6882, Tracor Analytic, Elk Grove, IL).

Quantitation of progesterone (Koligian et al., 1977) and testosterone (Chakraborty et al., 1984) was performed in a similar manner as the E<sub>1</sub> and E<sub>2</sub> assays, following diethyl ether extraction. In the progesterone RIA, the antibody (#337) was used at a dilution of 1:1,000 in 0.1% PBS-G. The radiolabelled hormone used in this assay was [1,2,5,7<sup>3</sup>H] progesterone (specific activity 80-110 Ci/mMol, Amersham). In the testosterone RIA, the antibody was incubated (48 hr at 4°C) with 4.0 ng/ml of dihydrotestosterone (DHT) in order to reduce the cross-reactivity of the antibody. The cross-reactivity of the testosterone antibody with DHT is approximately 70%. Following incubation with DHT, the cross-reactivity of the antibody with DHT is reduced to 10-15%. The radiolabelled hormone used in the assay was [1,2,5,7<sup>3</sup>H]testosterone (specific activity 80-105 Ci/mMol, Amersham). For both the progesterone and testosterone assays, standard solutions of progesterone or testosterone were run with each assay at concentrations between 0.01 to 1.0 ng/assay tube. The minimum amount of P or T detectable by these assays was 0.01 ng/assay tube. Duplicate aliquots (0.1 ml) of extracted samples were dried and incubated with the appropriate tritiated hormone (5 µCi/0.1 ml) and antibody for 2 hr at room temperature (final incubation volume was 0.2 ml). Following the incubation, the samples were placed on ice for 10 min and 0.5 ml of dextran-charcoal suspension

was added. The final steps of the progesterone and testosterone assays were the same as in E<sub>1</sub> and E<sub>2</sub> assays.

#### Luteinizing Hormone Assay:

Serum concentration of luteinizing hormone (LH) was determined with a double antibody radioimmunoassay specific for porcine LH (Chakraborty et al., 1973). Preparation of assay buffers are detailed in Appendix II. Standard solutions between the concentrations of 0.125 to 8.0 ng/tube were used to construct the standard curve. Minimal detectable level of LH in this assay system was approximately 0.15 ng/tube. Purified porcine LH (LER-786-3) was used as the standard and the radiolabelled hormone. The buffer system for LH assay was 0.1% PBS-G. Normal rabbit serum (NRS) was diluted 1:400 in 0.5M PBS-EDTA and was used to determine the degree of nonspecific binding. Iodination of the purified porcine LH was performed by the method of Greenwood et al. (1963). Iodinated LH was separated from free <sup>125</sup>I with a sephadex G-75 column. The first antibody was a rabbit anti-bovine LH gamma globulin (JJR#5) and was used at a dilution of 1:80,000 in NRS. The second antibody (sheep antirabbit gamma globulin, diluted 1:60 in PBS) was prepared as recommended by Niswender et al. (1969). Serum samples (0.2 ml) were incubated with 0.1% PBS-G (0.3 ml) and the first antibody (0.2 ml) for 48 hours. Next iodinated LH (30,000 cpm/0.1 ml) was added and incubation (24 hr) was continued. The incubation mixture was then allowed to incubate another 60-65 hr, following the addition of 0.2 ml of the second antibody. Final step of the assay was the addition of 1 ml of PBS to the samples and centrifugation (1000 g for 30 min). The supernatant was discarded and the pellet was counted (1 min/sample) in a gamma scintillation counter (Model 5160, Packard

Instrument Co., Downers Grove, IL).

#### Evaluation of Data:

A calculator (Cannon CP2020, Alexandria, VA) preprogrammed to perform regression analysis was utilized to determine the steroid and protein hormone concentrations of the unknowns. The calculated concentrations were multiplied by the appropriate dilution factor and reported as pg/ml or ng/ml of serum.

#### Steroid Hormone Receptor Assay

##### Preparation of Endometrial Samples:

Quantitation of endometrial estrogen and progesterone receptors (unoccupied), was performed as recommended by Rexroad (1981) and Rexroad et al. (1984). Endometrial tissue (1 g) was minced and 8 ml of Tris-EDTA buffer (TE) was added. The tissue was next homogenized with a Polytron (Brinkman Instruments, Westbury, NY). The homogenate was centrifuged (Sorvall, Wilmington, DE) at 800 g for 10 min. The supernatant was transferred into another chilled test tube and centrifuged again (11,000 g for 20 min). The supernatant (cytosolic fraction) was then diluted to 20 ml with TE buffer and then kept on ice until added to the receptor assays. Preparation of reagents and buffers are described in Appendix III. The pellet from the first centrifugation was used in the preparation of the nuclear fraction. The pellet was resuspended in 8 ml of TE buffer and centrifuged (800 g for 10 min). The supernatant was discarded and 8 ml of 0.4M KCl was added to the remaining pellet. The mixture was allowed to incubate for 1 hr at 4°C (mixture was vortexed every 15 min during this period). The KCl extracted pellet was next centrifuged (800 g for 10 min) and the supernatant (nuclear fraction)

was kept on ice, until assayed.

#### Estrogen Receptor Assay:

Tritiated estradiol - ([2,4,5,7-<sup>3</sup>H]estradiol, specific activity 90-100 Ci/mMol, New England Nuclear, Boston, MA) was the radioligand used in the estrogen receptor (cytosolic and nuclear) assay. The concentrations of tritiated estradiol ranged from 0.025nM to 0.4nM. The final incubation volume was 0.6 ml (0.2 ml of either the cytosol or nuclear fraction was added to the assay tubes) with the addition of appropriate amount of Tris-EDTA-DTT buffer (TED). Nonspecific binding of estradiol was determined by performing the incubation in the presence of 100-fold excess of unlabelled estradiol (Sigma Chemical Company, St Louis, MO), at each concentration of tritiated estradiol. The samples were allowed to incubate with the radioligand overnight (18-25 hr at 4°C). Following incubation, 1 ml of dextran-charcoal suspension was added and after an additional 8 min of incubation, the incubation mixtures were centrifuged (800 rpm for 10 min). The supernatant was decanted into scintillation vials and 8 ml of scintillation cocktail (Hydrofluor, National Diagnostics, Sommerville, NJ) was added. The samples were then counted in a beta counter (Mark III Model 6882, Tracor Analytic, Elk Grove Village, IL) for 2 min each.

#### Progesterone Receptor Assay:

Tritiated promegesterone (<sup>3</sup>H-R5020, specific activity 70-87 Ci/mMol, New England Nuclear, Boston, MA) was used as the radioligand in this assay. Concentrations of <sup>3</sup>H-promegesterone used, ranged from 0.125 nM to 2nM. Nonspecific binding of promegesterone was determined by performing the assay incubation in the presence of 100-fold excess of

radioinert promegesterone (New England Nuclear, Boston, MA). The progesterone receptor (cytosolic and nuclear) assay procedure was the same as described for the estrogen receptor assay.

#### Scatchard Analysis

The dissociation constant and the concentration of receptors were calculated by performing linear regression analysis of bound/free ratio versus bound hormone (Scatchard, 1949).

#### Protein Determination

Protein in the tissue preparations was quantitated by a colorimetric assay (Bradford, 1976) as supplied by Bio-Rad protein assay kit (Bio-Rad Chemical Division, Richmond, CA). The protein determination is based on the staining of proteins by Coomassie Brilliant Blue. Upon binding to proteins, the color of the Coomassie stain changes from red to blue. This color change is accompanied by a shift in the absorption maximum of the Coomassie stain from 465 nm to 595 nm, which is utilized to quantitate protein in samples.

The dye reagent was used at a 1:5 dilution in deionized water. Solutions of bovine serum albumin at concentrations of 0.2 to 1.2 mg/ml were used to construct the standard curve. Diluted dye reagent (5.0 ml) was added to aliquots (0.05 ml) of tissue preparation and allowed to incubate for 5 min. The optical density of standards and samples at 595 nm was measured by a spectrophotometer (Perkin-Elmer, Oak Brook, IL). The concentration of protein in samples was calculated from the standard curve and multiplied by the appropriate dilution factor. Protein concentrations were expressed in mg/ml.

### Statistical Analysis

Data from experiments are reported as mean  $\pm$  standard error of the mean (SEM). Two sample means were compared by Student's t-test and the level of significance was set at  $p < .05$  (Steel and Torrie, 1960). Data from several groups was analyzed by the least square analysis of variance and when a significant variance ratio (F value) was obtained, multiple comparison of means was performed by Student Newman-Keuls test (Steel and Torrie, 1960). The level of significance was specified at  $p < .05$ .

#### Description of Major Experiments:

##### Experiment I. Documentation of endometrial morphology in CEH and non-CEH sows.

In order to examine the overall appearance of the endometrium relative to information collected from other experiments, following sacrifice the uteri from eleven CEH and five non-CEH animals were examined and photographed. The objective of this experiment was to determine the changes in the gross morphology and histology of the uterus, associated with CEH. The uterus of CEH sows was classified as early or advanced CEH based on the size and distribution of the endometrial cysts. Uterine tissue specimens collected from CEH and non-CEH sows were stained with hemotoxylin and eosin so that the cellular morphology of the normal uterus could be compared with that from CEH animals. The age of sows with early CEH was not significantly different ( $p > .05$ ) from the non-CEH animals ( $35.5 \pm 8.8$  mo and  $23.9 \pm 3.1$  mo, respectively). Sows with advanced CEH were significantly older ( $p < .05$ )

than non-CEH sows, but not significantly different from sows with early CEH. The average age of the CEH sows was  $50.3 \pm 3.0$  mo.

Experiment II. Determination of hormonal profiles of CEH and non-CEH sows.

The objective of the experiment was to determine whether an imbalance in the serum concentrations of estrone ( $E_1$ ), estradiol ( $E_2$ ), progesterone (P), testosterone (T) and luteinizing hormone (LH) was associated with the occurrence of CEH. Although the hormone profile of and the length of the estrous cycle in SLA miniature sows had been established by a previous study (Howard et al., 1983), the occurrence of CEH in SLA miniature pigs was not known at the time of the previous investigation. Therefore a similar study was conducted and the animals were grouped according to whether or not they exhibited the hyperplastic condition.

Nine SLA miniature sows were assigned to CEH and non-CEH groups based on the evaluation of the uterus, by a midventral laparotomy. Five sows were found to exhibit the CEH condition and the remaining four sows had normal uteri. Sows in the CEH group were significantly older ( $p < .01$ ) than sows in the non-CEH group ( $52.8 \pm 6.1$  vs  $23.0 \pm 3.5$  mo, respectively). The body weight of CEH animals was significantly greater ( $p < .01$ ) than that of non-CEH sows ( $107.6 \pm 4.3$  vs  $84.9 \pm 6.2$  kg, respectively).

A synthetic progestin Altrenogest (Regu-Mate, Hoechst, Somerville, NJ), was orally administered ( $0.044$  mg/kg/day, mixed with feed) to both groups of animals for 14 days for the synchronization of estrous cycles. Following the withdrawal of progestin treatment, CEH and non-CEH

sows were checked once daily for signs of behavioral estrus. Behavioral estrus was defined as sexual receptivity of the sows to a test boar which was placed in their pens. Both groups of sows exhibited behavioral estrus approximately one week ( $8.3 \pm 0.6$  days) after the last day of progestin administration. The duration of behavioral estrus was similar in CEH and non-CEH sows ( $2.1 \pm 0.5$  and  $2.3 \pm 0.3$  days, respectively).

Daily collection of blood samples was initiated 48 hr following the withdrawal of Altrenogest treatment and continued throughout the period of detectable behavioral estrus. Thereafter blood samples were obtained on alternate days. Daily blood collection was resumed approximately 21 days after the first estrus period, since the length of the estrous cycle had been previously determined to be 21 days (Howard et al., 1983). Serum samples were stored at  $-20^{\circ}\text{C}$  until assayed.

Steroids were extracted from serum samples and assayed for  $\text{E}_1$ ,  $\text{E}_2$ , P and T by RIA. Recovery of the steroid hormones from the extraction procedure ranged between 90.7 to 99.5%. Intra-assay coefficients of variation for  $\text{E}_1$ ,  $\text{E}_2$ , P and T were 7.8%, 6.9%, 8.1% and 10.0%, respectively. Interassay coefficients of variation were 7.9%, 11.7%, 9.8% and 11.4% for  $\text{E}_1$ ,  $\text{E}_2$ , P and T, respectively. Serum concentrations of LH were measured by a specific porcine LH RIA, in a single assay. The LH intra-assay coefficient of variation was 5.3%.

Experiment III. Evaluation of endometrial estrogen and progesterone receptors during the luteal phase of the estrous cycle of CEH and non-CEH sows.

Since the effects of hormones are normally exerted on their target tissues through the interaction with their receptors, it was of interest to quantitate endometrial estrogen and progesterone receptors

in CEH and non-CEH sows. Such data would provide information on the possible role of sex steroid hormone receptors in endometrial hyperplasia. Due to the limited availability of SLA miniature sows for this study and the short length of the estrus period (average 3.1 days, Howard et al., 1983), the populations of endometrial estrogen and progesterone receptors were examined only during the luteal phase of the estrous cycle.

Endometrial tissue specimens were collected from seven CEH and seven non-CEH sows at necropsy. The endometrial tissue samples were stored at  $-80^{\circ}\text{C}$  until processed for the quantitation of cytoplasmic and nuclear estrogen and progesterone receptors. The average age of the CEH sows ( $45.1 \pm 1.2$  mo) was significantly greater ( $p < .01$ ) than the mean age of non-CEH sows ( $10.2 \pm 1.2$  mo).

Experiment IV. Investigation of the effects of 17- $\beta$ -estradiol, progesterone, and clomiphene citrate on the development of cystic endometrial hyperplasia.

Since cystic endometrial hyperplasia occurs spontaneously in miniature swine, the hormonal factors if any, involved in the etiology of CEH are unknown. The objectives of this experiment were to determine if CEH in miniature swine may be induced by exogenous estrogen administration and if the administration of clomiphene citrate or progesterone would alleviate this condition. In addition, sex steroid hormone receptors were quantitated to determine whether any histological changes observed could be related to changes in the concentrations of endometrial sex steroid hormone receptors. Non-CEH sows were assigned to placebo or 17- $\beta$ -estradiol treatment and CEH sows were assigned to clomiphene citrate or progesterone therapy. Furthermore, non-CEH sows were bilaterally ovariectomized in order to remove any

modifying influence of endogenous sex steroid hormones on the response to hormone treatment. Due to the limited number of animals (15 sows) available for the in vivo study, a group of intact CEH sows with placebo implants could not be maintained.

A midventral laparotomy was performed on 15 sows, to distinguish CEH sows from non-CEH sows. Sows with normal uteri were bilaterally ovariectomized, at the time of the laparotomy. Eight sows exhibited CEH and seven sows had normal uteri. The ages of the CEH sows ranged from 39 to 78 mo, whereas the ages of the non-CEH sows ranged from 12 to 32 mo. The CEH sows were significantly older ( $p < .01$ ) than the animals in the non-CEH group ( $50.9 \pm 4.4$  vs  $18.7 \pm 3.0$  mo, respectively). As a result of the age differential, the mean body weight of CEH sows ( $108.5 \pm 3.3$  kg, range was 98.4 to 122.5 kg) was significantly greater ( $p < .01$ ) than that of non-CEH animals ( $76.5 \pm 5.5$  kg, range was 58.1 to 96.6 kg). The sows were allowed two weeks to recover from surgery, before assignment to treatment groups.

The seven non-CEH sows were assigned at random to placebo and 17- $\beta$ -estradiol treatment groups. The average age and body weight of the three sows in the placebo group were  $17.0 \pm 3.6$  mo and  $74.5 \pm 11.5$  kg. The four non-CEH sows receiving 17- $\beta$ -estradiol had a mean age of  $20.0 \pm 4.9$  mo and the average body weight was  $78.1 \pm 6.3$  kg. Of the eight CEH sows, four sows were assigned to the progesterone treatment group. Sows in this group had a mean age of  $55.8 \pm 8.3$  mo and the average body weight was  $105.1 \pm 3.9$  kg. CEH sows in the clomiphene citrate group ( $n=4$ ), were  $46.0 \pm 2.8$  mo old and weighed  $111.8 \pm 5.2$  kg.

All the treatments were administered to the sows by means of pelletized products (Innovative Research of America, Rockville, MD). This method of administration of the treatments was chosen for its reliable release rate and to ensure fairly constant levels of the respective treatments in the circulation of the animals. The four treatment groups in this study were placebo, 17- $\beta$ -estradiol, clomiphene citrate and progesterone. Duration of all treatments was 90 days and the pellets were designed for 45-day release. Thus the animals were re-implanted with additional pellets after the first 45-day period.

All the pellets were designed for use in animals with body weights between 80 to 90 kg. The placebo pellet consisted of only the matrix components (cholesterol, methyl cellulose and  $\alpha$ -lactose) and each animal was implanted subcutaneously with nine pellets. The 17- $\beta$ -estradiol pellets were designed to maintain 100-200 pg/ml of estradiol (60 mg/pellet, 3 pellets/animal) in the circulation. The desired level of progesterone in the circulation from the progesterone pellets was 60 ng/ml (250 mg/pellet, 9 pellets/animal). These specified concentrations of estradiol and progesterone desired to be released by the pellets were approximately three times the peak serum concentrations, observed during an estrous cycle. Based on a previous study that examined the uterotrophic response to clomiphene citrate (Poteat et al., 1971), the clomiphene citrate pellets were designed to release a circulating level of 40-50 ng/ml (225 mg/pellet, 9 pellets/animal).

Implantation of the pellets was performed under intravenous barbiturate anesthesia (17.6 mg/kg, Bio-Tal). The pellets were placed subcutaneously in the medial shoulder region of sows. Dexon was used to suture the skin incision and an antibiotic (nitrofurazone, Nitro-Fur Puffer, Life Sciences Products) was topically applied to the incision site.

Collection of blood samples from the sows in the treatment groups was initiated one week after the implantation of the various treatment pellets. Each of the treatment groups were separated into two groups and blood samples were obtained alternately from these two groups on a weekly basis throughout the study (total of 12 weeks). Serum concentrations of  $E_2$  and P were measured by RIA every two weeks to monitor the levels of the treatments maintained by the pellets.

At the end of the 90-day treatment period, all the animals were sacrificed. Blood samples were collected prior to sacrifice. Immediately after sacrifice, uteri were removed and weighed. The gross morphology of the uterus and the endometrium was photographically recorded. Endometrial tissue for histological analysis and quantitation of estrogen and progesterone receptors were obtained at necropsy. Pituitary glands were also obtained at this time. Since a direct method of measuring serum concentration of clomiphene citrate was not available in the laboratory, pituitary and serum concentration of LH were measured at the end of the study to examine the effectiveness of the clomiphene citrate pellets. In addition, it was of interest to examine how the various treatments would affect the pituitary content of luteinizing hormone. This information would indicate whether the effect(s) of a specific treatment might be mediated through the pituitary. The serum concentrations of  $E_2$ , P and LH were determined by RIA.

Recovery of steroids from the extraction procedure ranged between 91.5 and 98.2%. Intra-assay coefficients of variation of  $E_2$  and P were 7.3% and 6.5%, respectively. Interassay coefficients of variation were 8.9% and 7.3% for  $E_2$  and P. The intra- and interassay coefficients of variation of LH were 6.8% and 8.03%, respectively.

## Chapter 4

### PRELIMINARY STUDIES

#### Steroid Hormone Content of Cyst Fluid and Serum from CEH and non-CEH Animals

An initial experiment was performed to examine whether a hormonal imbalance was associated with the occurrence of cystic endometrial hyperplasia in SLA miniature pigs. Blood samples were collected from seven CEH and three non-CEH sows at necropsy. The sows were sacrificed during the luteal phase (day 10 or day 14) of the estrous cycle. In CEH sows, cyst fluid was also aspirated from endometrial cysts of various sizes (>10 mm to 50 mm in diameter). The age of the sows ranged between 5 mo and 6 yr old. Steroids were extracted from cyst fluid and serum samples and concentrations of  $E_1$ ,  $E_2$ , P and T were measured by RIA. Intra-assay coefficients of variation for  $E_1$ ,  $E_2$ , P and T were 10.7%, 9.9%, 14.2% and 14.3%, respectively.

The concentrations of the steroid hormones in serum and cyst fluid are listed in Table 1. The steroid hormone concentrations in cyst fluid were not different ( $p > .05$ ) between cysts of various sizes (data not shown). Serum concentrations of  $E_1$ ,  $E_2$ , P and T in CEH sows were not significantly different from those of normal sows. Furthermore, cyst fluid contained significantly lower ( $p < .05$ ) concentrations of steroid hormones when compared to the serum levels of both CEH and non-CEH animals. However the data of this pilot study were limited to only two days of luteal phase of the estrous cycle and thus any differences which may have existed at other times would not have been detected.

Table 1. Comparison of serum concentrations of estrone, estradiol, progesterone and testosterone in cyst fluid and serum from CEH and non-CEH animals. Samples of cyst fluid and serum were obtained from control and CEH pigs during days 10 and 14 of the estrous cycle. Results are shown as mean  $\pm$  SEM and values bearing different superscripts are significantly different ( $p < .05$ ) from each other.

Steroid Concentrations				
Animals	Estrone (pg/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)	Testosterone (ng/ml)
<u>CEH</u>				
Cyst Fluid	17.5 ± 1.3 <sup>a</sup>	11.9 ± 0.6 <sup>a</sup>	2.8 ± 0.1 <sup>a</sup>	0.1 ± 0.006 <sup>a</sup>
Serum	26.1 ± 3.9 <sup>b</sup>	29.2 ± 3.1 <sup>b</sup>	15.2 ± 0.9 <sup>b</sup>	0.2 ± 0.03 <sup>b</sup>
<u>non-CEH</u>				
Serum	29.5 ± 1.4 <sup>b</sup>	28.3 ± 5.7 <sup>b</sup>	13.7 ± 2.3 <sup>b</sup>	0.2 ± 0.04 <sup>b</sup>

### Steroid Hormone Profiles of CEH and non-CEH Sows

Three CEH sows and three non-CEH sows were housed outdoors in a covered enclosure (approximately 6 m<sup>2</sup>/animal). Sows were fed Purina Complete Sow Chow (1.0-1.5 kg/day, Ralston Purina Co., St. Louis, MO) twice daily. Water was available ad libitum. A synthetic progestin, Altrenogest (Regu-Mate, Hoechst, Sommerville, NJ) was orally administered (0.044mg/kg/day, mixed with feed) for 14 days to synchronize estrous cycles. Pregnant mare's serum gonadotropin (PMS, 1200 I.U., i.m.) was administered to the animals, following the last day of progestin treatment. The sows were then given human chorionic gonadotropin (HCG, 500 I.U., i.m.) 80 hr following PMS administration. These gonadotropins were given to the sows to control the time of and also to enhance ovulation, as a part of an embryo collection study. The sows were determined to be in estrus when sexually receptive to a test boar.

Blood samples were collected twice daily through an indwelling jugular catheter. Steroids were extracted from serum samples and assayed for E<sub>1</sub>, E<sub>2</sub>, P and T by RIA. Intra-assay coefficients of variation for E<sub>1</sub>, E<sub>2</sub>, P and T were 10.0%, 12.7%, 8.3% and 14.0%, respectively. Interassay coefficients of variation were 11.0%, 9.9%, 15.0% and 13.3% for E<sub>1</sub>, E<sub>2</sub>, P and T. Serum concentrations of estrone, estradiol and progesterone of individual non-CEH and CEH sows are represented in Figures 1 and 2, respectively.

Behavioral estrus was detected approximately two weeks after the withdrawal of progestin therapy, in both non-CEH and CEH sows. The duration of estrus was two days in both of the animals. In agreement with similar superovulation studies performed by Diehl (unpublished data), serum concentrations of estradiol and progesterone did not appear to

be different between the non-CEH and CEH sows. Preliminary analysis of serum concentration of estrone did not indicate any difference in the level of this hormone between CEH and non-CEH sows. In addition, serum concentrations of testosterone (data not shown) was found to be between 0.15 and 0.40 ng/ml, in both normal and CEH sows. As this initial investigation utilized exogenous gonadotropins to induce superovulation this could have resulted in hormone concentrations different from that during a non-treated estrous cycle. In order to address this issue, a subsequent study was conducted to examine the serum concentrations of the sex steroid hormones during a synchronized estrous cycle of CEH and non-CEH animals not treated with exogenous gonadotropins.

Figure 1. Serum concentrations of estrone (□--□), estradiol (○—○) and progesterone (▲—▲) during and following synchronization of estrus in a non-CEH miniature sow (#5-5). Altrenogest was given orally from days 2 to 16; PMS and HCG were given at times indicated.

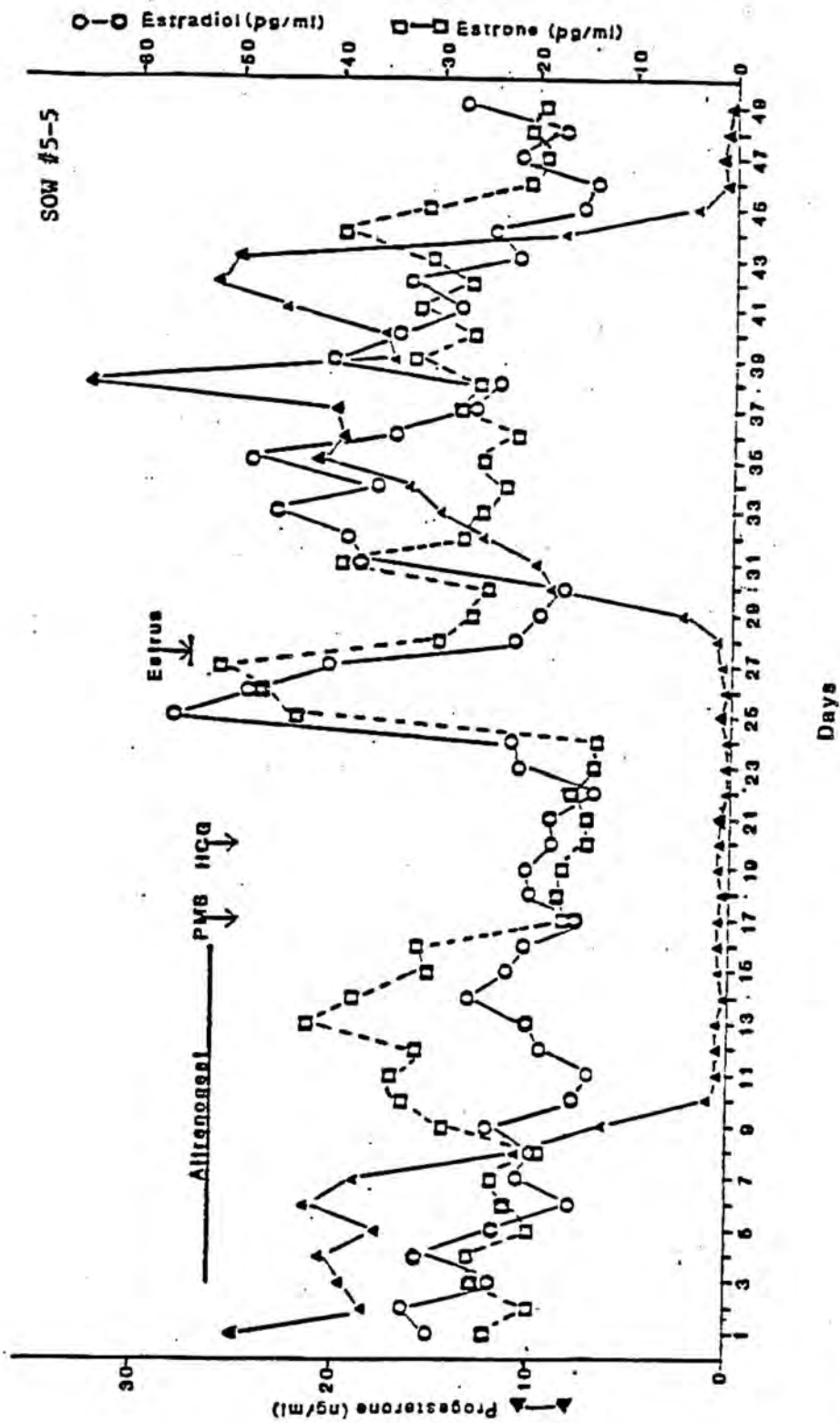
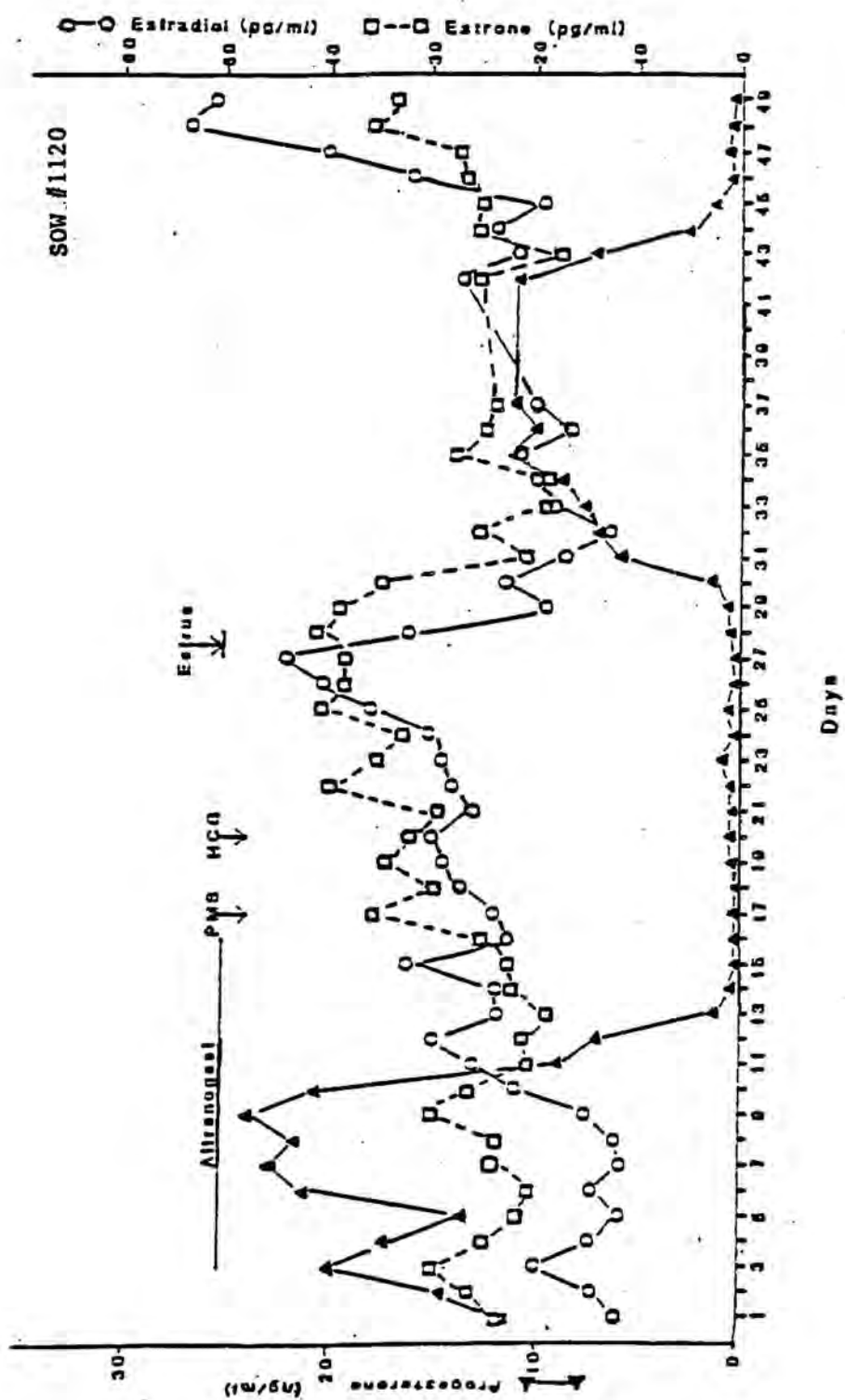


Figure 2. Serum concentrations of estrone ( $\square--\square$ ), estradiol ( $\circ--\circ$ ) and progesterone ( $\blacktriangle--\blacktriangle$ ) during and following synchronization of estrus in a CEH miniature sow (#1120). Altrenogest was given orally from days 2 to 16; PMS and HCG were given as indicated.



Electrophoretic Analysis of Serum, Uterine Flushings, and Endometrial Cyst Fluid from CEH and non-CEH Sows

The composition of serum and uterine flushings from non-CEH sows, and serum and endometrial cyst fluid from CEH sows, were analyzed by discontinuous gel electrophoresis. A primary objective of this preliminary investigation was to determine if any differences were present in electrophoretic patterns of serum samples from CEH and non-CEH animals. Electrophoretic analysis of serum samples could thus be a convenient method of differentiating between non-CEH and CEH animals. A difference in the nature of protein content of the endometrium between normal women and women with endometrial hyperplasia was demonstrated by Ciocca et al. (1985). Based on this information, the uterine fluid from CEH and non-CEH sows was examined to identify any protein differences associated with CEH.

Endometrial cyst fluid and uterine flush samples were diluted 1:4 with sample buffer containing mercaptoethanol. The dilution of serum samples with this same sample buffer resulted in the precipitation of proteins in the samples. Thus serum samples were diluted 1:100 with mercaptoethanol-free sample buffer.

Electrophoretic patterns of serum from non-CEH and CEH sows were found to be similar (Figure 3). The protein constituents of uterine flushings and endometrial cyst fluid differed from that of serum samples. Furthermore, endometrial cyst fluid was found to contain a protein between the molecular weights of 31K and 45K, which was not present in the uterine flushings nor in serum from either type of sow.



Figure 3. SDS Polyacrylamide gel electrophoretic patterns of uterine flushings from normal sows, endometrial cyst fluid from CEH sows, and serum from both types of sows. Arrow indicates the location of the protein which appears to be unique to cyst fluid.

Porcine uterine secretions have been previously reported to contain a purple protein (Murray et al., 1972). This protein was recovered in uterine flushings collected on days 12 and 16 of the estrous cycle and was naturally purple in color. Chen et al. (1975) utilized immunofluorescence to further characterize this protein. Uterine tissue showed varying intensities of fluorescence throughout the estrous cycle. Fluorescence was associated with endometrial, surface epithelium and glandular epithelium on days 0, 3 and 6 of the estrous cycle. On day 15, fluorescence was no longer visible in endometrial surface or glandular epithelium and glandular lumen. However, a high intensity of fluorescence was exhibited by endometrial stroma. By day 18, fluorescence in the endometrial stroma was faint. When animals were ovariectomized and treated with estrogen only, there was no evidence of the purple protein. On the other hand, there was readily detectable fluorescence in the glandular epithelium of ovariectomized animals that were administered estrogen and progesterone or progesterone alone. These investigators suggested that estrogen may initiate synthesis of the purple protein, but it is mainly progesterone which regulates the synthesis and secretion of the purple protein.

Another study by Bazer et al. (1975) found that a purple protein was also present in allantoic fluid of pregnant pigs. Uterine flushings were collected on day 15 of the estrous cycle, when progesterone levels are high. Allantoic fluid was collected from pigs throughout the gestation period. The purple protein found in uterine flushings and in allantoic fluid were both found to have similar electrophoretic properties and have a molecular weight of 32K. It was proposed that the purple protein is synthesized by endometrial glands and then trans-

ported into allantoic fluid. This study provided further evidence of regulation of purple protein by progesterone.

Although the protein which appears unique to cyst fluid has a molecular weight between 31k and 45K, it does not appear to be the purple protein found in standard-sized pigs. Mainly the uterine flushings and cyst fluid were collected on days 4, 9 and 15 of the estrous cycle and there was no detectable purple color in these samples. Moreover, the electrophoretic pattern of uterine flushings did not contain any protein bands with a molecular weight near 30K.

Since the electrophoretic pattern of serum from CEH and non-CEH sows were similar, electrophoretic analysis was found to be of little diagnostic value in predicting the presence of CEH. However the protein found to be unique to endometrial cyst fluid should be considered in future investigations of CEH in SLA miniature sows.

## Chapter 5

### RESULTS

#### Experiment I. Documentation of endometrial morphology in CEH and non-CEH sows.

##### Gross Morphology

Examples of a normal uterus and endometrium are shown in Figure 4. The outer layer of the uterus was smooth in texture and the uterine horns were not turgid (Panel A). The ovaries were normal in appearance and the primary structure included several corpora lutea. The endometrium was firm and uniformly red in color (Panel B).

The outer layer of an uterus from a CEH sow is usually similar in appearance to a normal uterus (Panel A, Figure 5). However, the ovaries contained cystic follicles. The presence of cystic follicles was occasionally observed in sows with CEH. On the other hand, cystic follicles also occur in normal sows. Upon exposure of the uterine lumen, the endometrium was observed to be in the early stages of CEH (Panel B). The endometrium was light brown and blotchy in color. Few localized endometrial cysts (<10 mm in diameter) were present.

An endometrium in the advanced stages of CEH usually contained numerous endometrial cysts, which were distributed throughout the entire layer (Panel A, Figure 6). The endometrial cysts were either translucent or opaque. Furthermore, the cyst fluid was clear or slightly yellow in color. Most of the cysts were round and they ranged in size between 10 and 40 mm in diameter. Some of the endometrial cysts near the ovarian end of the uterine horns, were approximately 50 mm in diameter (Panel B).

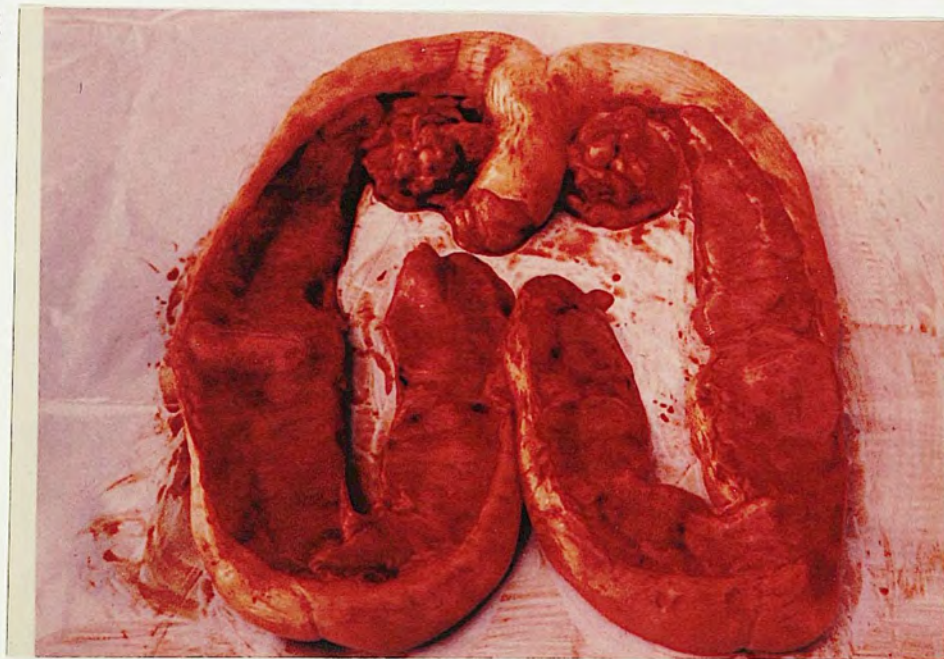
**A****B**

Figure 4. The uterus (Panel A) and endometrium (Panel B) from a normal (non-CEH) sow.



Figure 5.- The uterus (Panel A) and endometrium (Panel B) from a sow exhibiting changes associated with the early stages of the development of CEH.

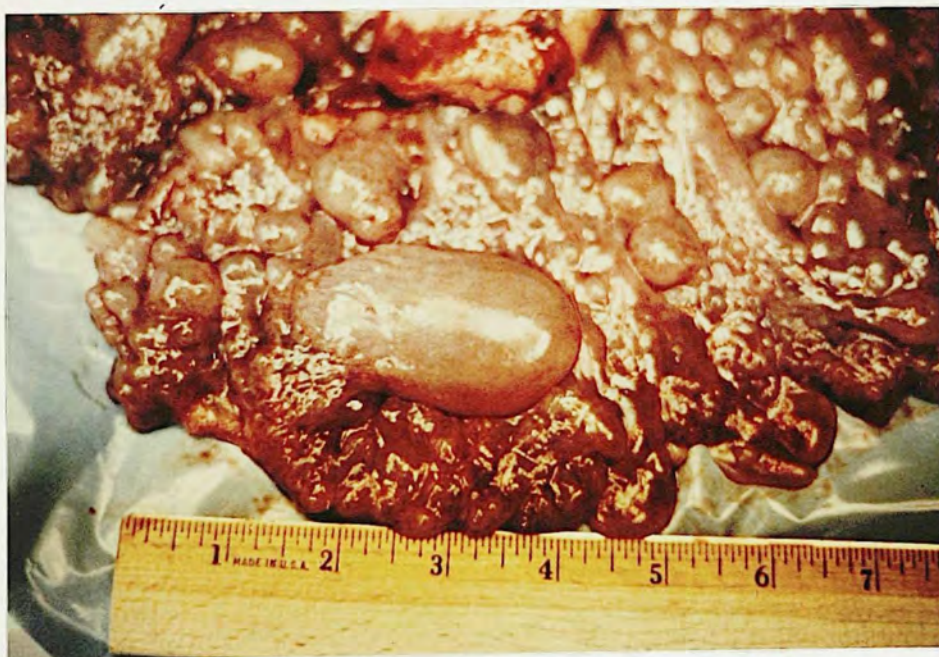
**A****B**

Figure 6. Cystic changes of the endometrium associated with the advanced stages of CEH (Panel A). Some cysts were 50 mm in diameter (Panel B).

### Histological Analysis

The histology of normal and hyperplastic endometria was examined with hemotoxylin and eosin staining, using light microscopy. In a normal endometrium, the surface epithelium of the endometrium lines the uterine lumen (Panel A, Figure 7). The endometrium can be further histologically classified into the stratum functionalis and the stratum basalis and these two layers were clearly distinguishable from each other. Endometrial glands were round in shape and uniformly distributed throughout the endometrial layer. Blood vessels were also found within the endometrial layer. On the other hand, the myometrium was composed of a smooth muscle fibers, but contained little vasculature (Panel B).

In the early stages of the development of CEH, the surface epithelium, stratum functionalis and stratum basalis were intact and distinct (Panel A, Figure 8). However, there appeared to be an increase in the number of endometrial glands and these glands were grouped together. The glands were also dilated and coiled or round in structure. Despite the presence of small endometrial cysts, the endometrium remained intact (Panel B).

During the advanced stages of the development of CEH, the endometrium was distorted due to the presence of large cysts (Panel A, Figure 9). The distinction between the stratum functionalis and stratum basalis was lost. There was also a further increase in the number of, as well as in the clustering endometrial glands. The endometrial glands were coiled or round and dilated and some of the glands had formed buds which protruded into the endometrial stroma. While the endometrial layer had become completely disrupted due to the infiltration of cysts, the myometrial layer was found to remain unaffected (Panel B).

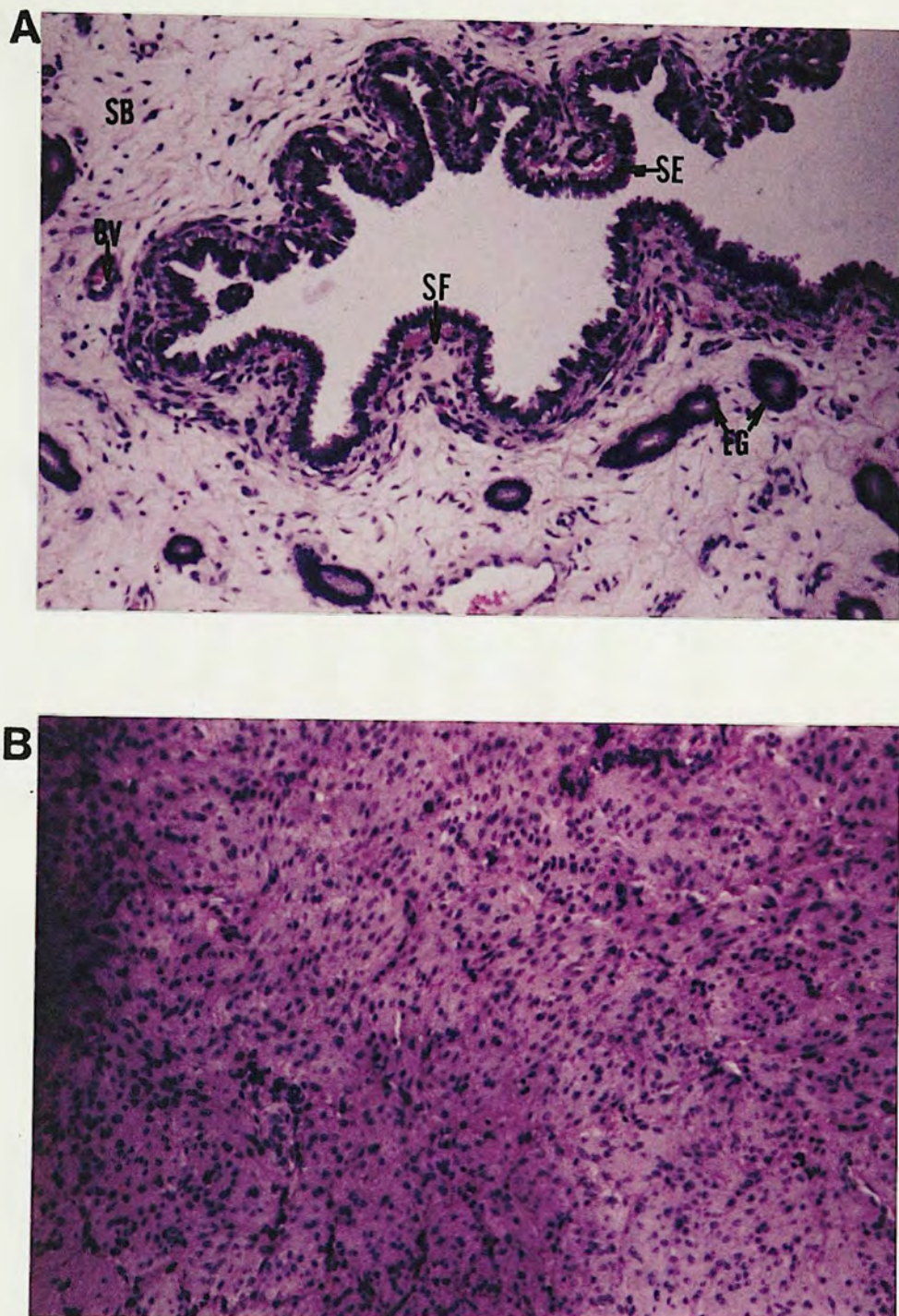


Figure 7. Histology of normal endometrium (Panel A, 100x) and myometrium (Panel B, 100x). The endometrium contains surface epithelium (SE), stratum functionalis (SF), stratum basalis (SB), endometrial glands (EG) and blood vessels (BV). The myometrium is composed of smooth muscle fibers.

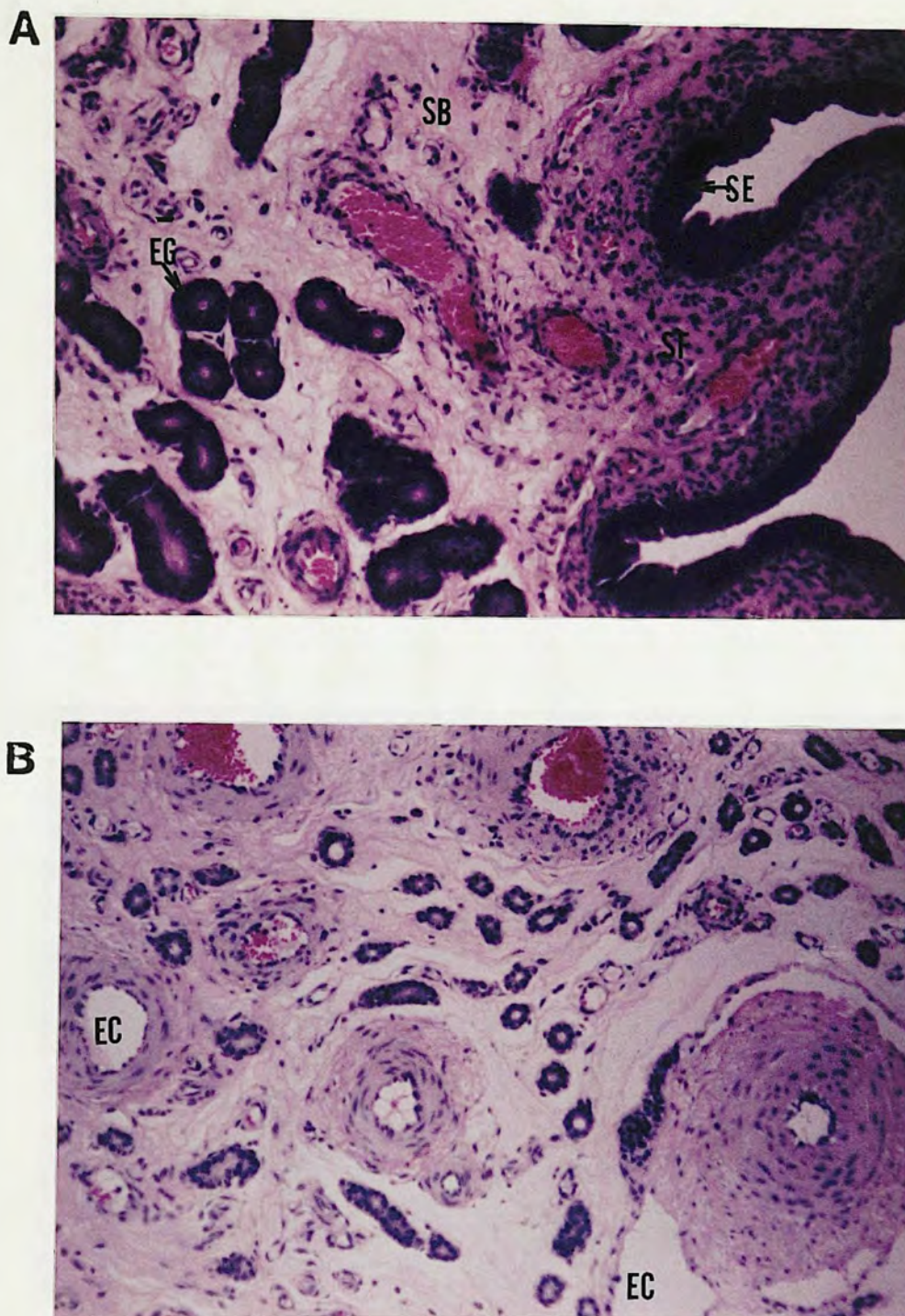


Figure 8. Histology of the endometrium during the early stages of development of CEH. The surface epithelium (SE), stratum functionalis (SF), stratum basalis (SB) remain intact, but there is an increase in the number of glands (EG), which have started to group together (Panel A, 200x). Small endometrial cysts (EC) were present in the endometrium (Panel B, 100x).

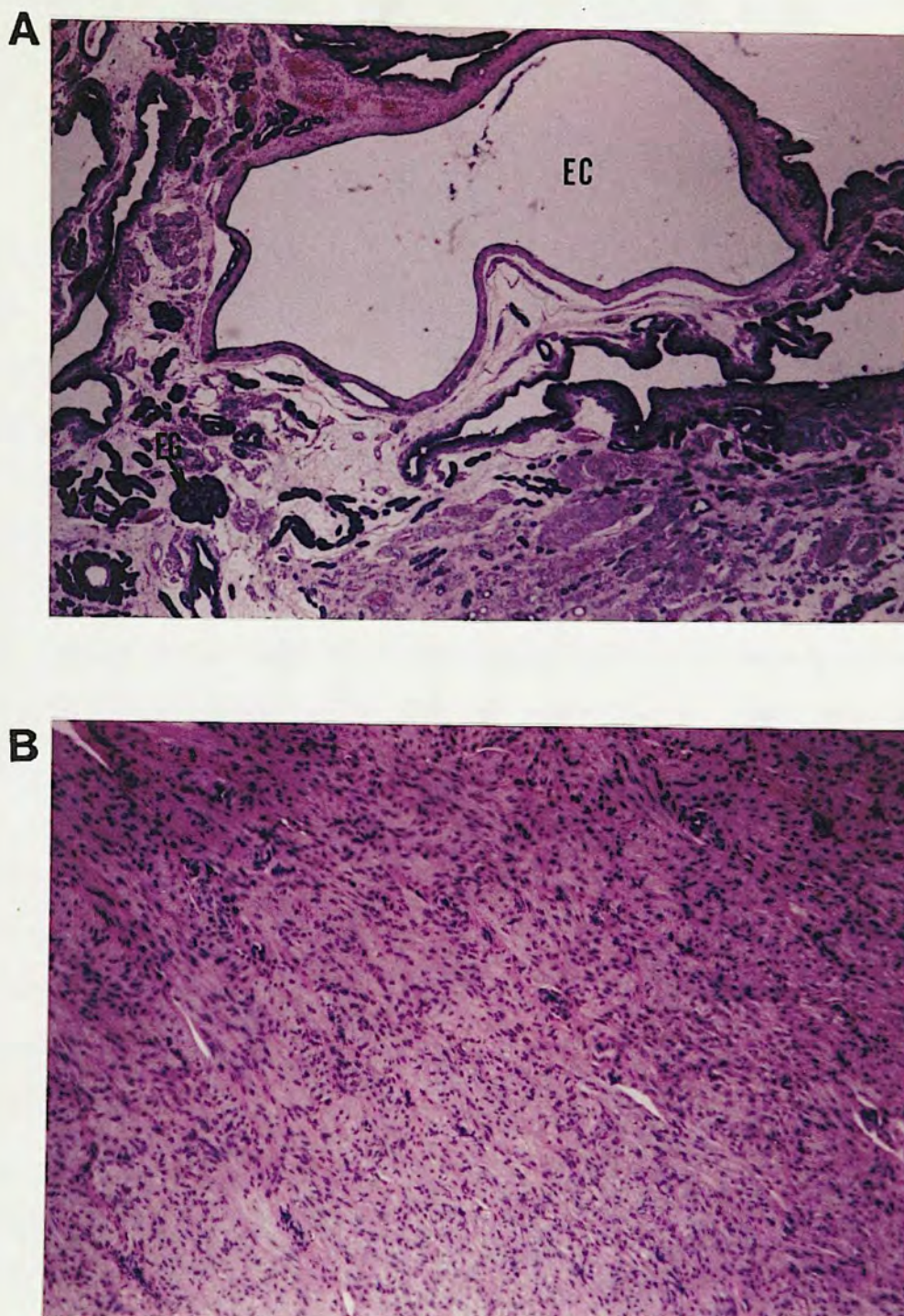


Figure 9. Histology of the endometrium and myometrium during the advanced stages of the development of CEH. The endometrial layer was completely distorted due to the presence of large endometrial cysts (EC) and further clustering of glands (EG) was evident (Panel A, 40x). However the myometrium remained unaffected (Panel B, 100x).

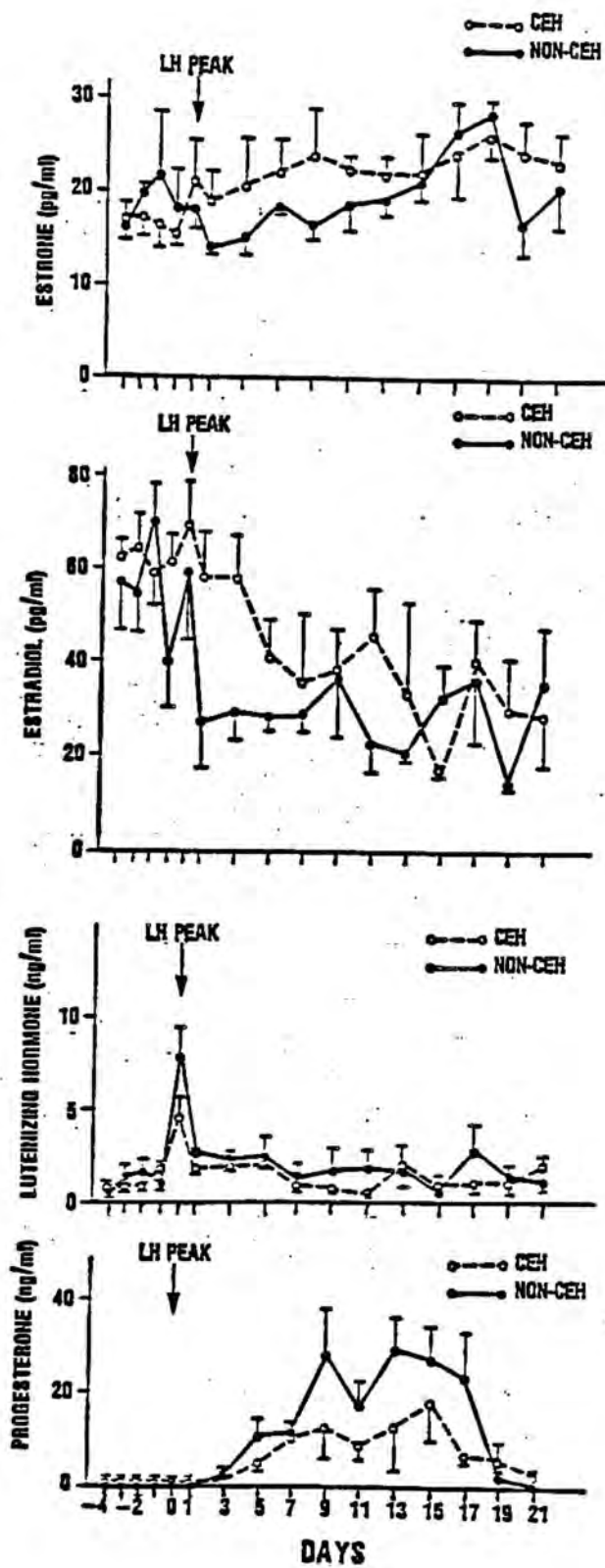
Experiment II. Determination of hormonal profiles of CEH and non-CEH sows.

Mean Hormonal Profiles

The mean hormonal profiles of  $E_1$ ,  $E_2$ , LH and P for CEH and non-CEH sows are represented in Figure 10. Data of the serum concentrations of sex steroid hormones were standardized to the day of the preovulatory LH surge (day 0). The general temporal relationships between the hormones were similar in both groups of sows. In CEH sows the mean serum estradiol concentration reached a peak level ( $69.6 \pm 8.9$  pg/ml), concurrently with the LH peak. The mean peak level of estradiol ( $70.0 \pm 8.1$  pg/ml) in non-CEH sows was detected 48 hr prior to the LH peak. There was no significant difference between the mean peak serum concentration of estradiol in CEH and non-CEH sows. Mean peak level of LH of the CEH group was found to be significantly lower ( $p < .05$ ) than that of the non-CEH group ( $4.7 \pm 0.9$  and  $7.9 \pm 1.5$  ng/ml, respectively) based on once daily blood collection during the period of estrus. In both groups of sows, mean serum concentration of progesterone began to rise from nadir levels on day 3 and remained elevated until day 15 in CEH sows and day 17 in non-CEH sows. Thereafter, there was an abrupt decline in the serum concentration of progesterone in both groups of sows. The mean serum level of testosterone (data not shown), varied between 0.15 and 0.50 ng/ml throughout the estrous cycle of both CEH and non-CEH sows.

During days -3 to 3 there were no significant differences found in the mean levels of  $E_1$ ,  $E_2$ , LH, P and T, between the CEH and non-CEH groups. Mean levels of these hormones and the respective ranges observed during days -3 to 3 of the estrous cycle of CEH and non-CEH sows, are listed in Table 2.

Figure 10. The mean hormonal profiles of estrone, estradiol, luteinizing hormone (LH) and progesterone during the synchronized estrous cycle of CEH and non-CEH sows. Arrow indicates the day of the LH peak.



Hormone	Animals	
	<u>CEH</u>	<u>non-CEH</u>
Estrone (pg/ml)	18.6 ± 1.2 (15.7 - 21.5)	18.2 ± 1.3 (14.5 - 22.0)
Estradiol (pg/ml)	62.1 ± 3.1 (58.0 - 69.6)	50.7 ± 5.3 (27.7 - 70.0)
Progesterone (ng/ml)	1.3 ± 0.2 (0.8 - 1.8)	0.9 ± 0.5 (0.3 - 3.9)
Testosterone (ng/ml)	0.3 ± 0.04 (0.1 - 0.4)	0.2 ± 0.02 (0.2 - 0.3)
Luteinizing Hormone (ng/ml)	2.1 ± 0.3 (0.9 - 4.7)	2.9 ± 0.6 (1.0 - 7.9)

Table 2. Comparison of serum concentrations of estrone, estradiol, progesterone, testosterone and luteinizing hormone during days -3 to 3 of the estrous cycle of CEH and non-CEH sows. Values are expressed mean ± SEM. Values within parenthesis are the respective ranges.

During days 5 to 17 of the estrous cycle no differences ( $p > .05$ ) in the mean serum concentrations of  $E_1$ ,  $E_2$ , T and LH were observed between CEH and non-CEH sows. Mean serum progesterone concentration ( $10.2 \pm 2.0$  ng/ml) in CEH sows was, however, significantly lower ( $p < .01$ ) compared to that of non-CEH sows ( $21.1 \pm 2.8$  ng/ml). The mean serum concentrations of  $E_1$ ,  $E_2$ , P, T and LH and the respective ranges of these hormones observed during days 5 to 17 of the estrous cycle are listed in Table 3.

#### Individual Hormonal Profiles

Examples of the types of variability observed in individual profiles of sows in the CEH and non-CEH groups are presented in Figures 11 and 12. In CEH sow #995, behavioral estrus was exhibited from days -1 to 0 of the estrous cycle (Figure 11). Serum concentrations of estrone varied between 12.8 and 28.1 pg/ml between days -3 to 3 and between 14.2 and 20.9 pg/ml, thereafter. Serum estradiol reached a peak concentration of 86.4 pg/ml, 36 hr prior to the day of the surge in luteinizing hormone. A second rise in serum level of estradiol (84.0 pg/ml) was detected on the same day as the surge in LH (2.7 ng/ml). Thereafter serum concentrations of estradiol varied between 42.1 pg/ml and 16.0 pg/ml until day 19. Serum progesterone concentrations varied only between 5.5 and 7.0 ng/ml during days 5 to 17 of the estrous cycle.

In non-CEH sow #503.6, behavioral estrus was observed during days 0 to 2 of the estrous cycle (Figure 12). Serum concentrations of estrone varied between 9.2 and 18.8 pg/ml between days -3 to 3 and 17.0 and 31.0 pg/ml, thereafter. Peak serum estradiol concentration of 74.8 pg/ml was detected four days prior to the LH peak (7.1 ng/ml). There

was a gradual fall in the serum level of estradiol from days -2 to 11. Serum level of progesterone started to rise from nadir on day 1, to a maximal level of 26.2 ng/ml by day 11. Progesterone concentrations remained elevated until day 17. By day 21 the serum level of progesterone had returned to nadir. On day 17 of the cycle, a second estradiol peak of 66.0 pg/ml was observed to be accompanied by a LH peak (4.3 ng/ml). However behavioral estrus was not detected during this time. Hormonal profiles ( $E_1$ ,  $E_2$ , LH and P) from individual sows in the CEH and non-CEH groups are presented in Appendixes IV and V, respectively.

Hormone	Animals	
	<u>CEH</u>	<u>non-CEH</u>
Estrone (pg/ml)	23.8 ± 1.2 <sup>a</sup> (22.0 - 26.8)	23.9 ± 1.8 <sup>a</sup> (16.7 - 29.3)
Estradiol (pg/ml)	36.6 ± 3.3 <sup>a</sup> (17.8 - 46.1)	28.8 ± 2.8 <sup>a</sup> (20.9 - 37.5)
Progesterone (ng/ml)	10.2 ± 2.0 <sup>a</sup> (5.8 - 18.9)	21.6 ± 2.9 <sup>b</sup> (11.7 - 30.7)
Testosterone (ng/ml)	0.3 ± 0.03 <sup>a</sup> (0.2 - 0.5)	0.4 ± 0.04 <sup>a</sup> (0.3 - 0.5)
Luteinizing Hormone (ng/ml)	1.4 ± 0.2 <sup>a</sup> (0.6 - 2.3)	1.9 ± 0.4 <sup>a</sup> (0.6 - 2.9)

Table 3. Comparison of serum concentrations of estrone, estradiol, progesterone, testosterone and luteinizing hormone during days 5 to 17 of the estrous cycle of CEH and non-CEH sows. Values are expressed as mean ± SEM and values bearing different superscripts across rows are significantly different ( $p < .01$ ) from each other. Values within parenthesis are the respective ranges.

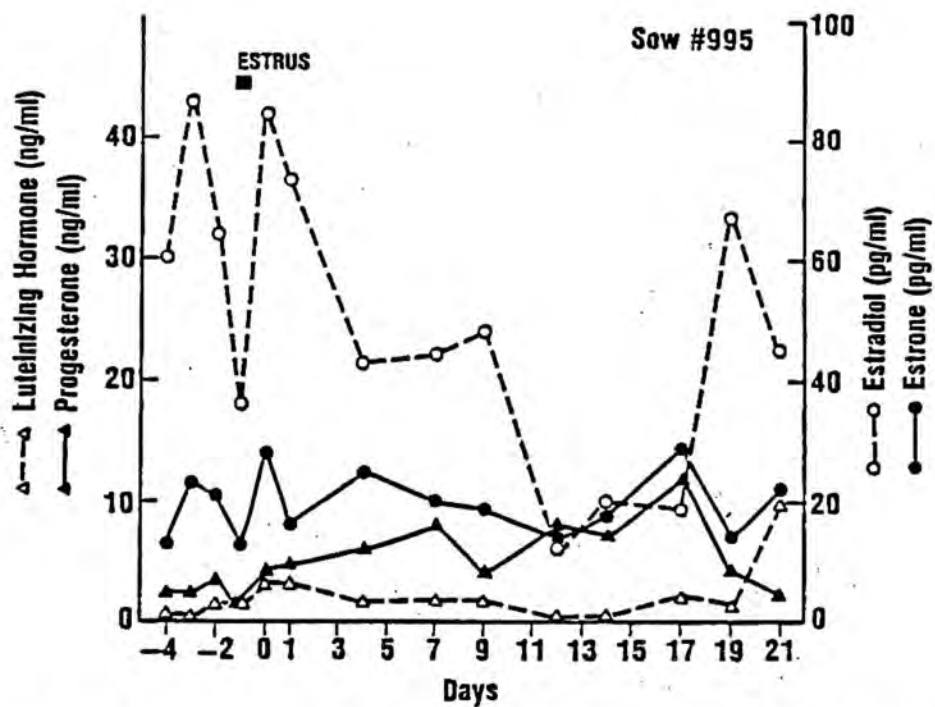


Figure 11. Serum concentrations of estrone, estradiol, luteinizing hormone and progesterone during the synchronized estrous cycle of CEH sow #995.

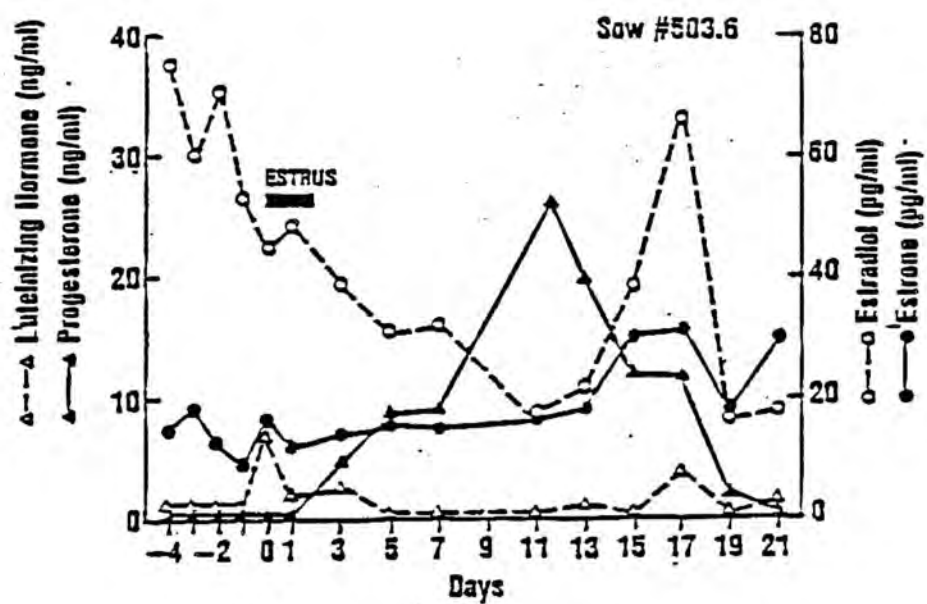


Figure 12. Serum concentrations of estrone, estradiol, luteinizing hormone and progesterone during the synchronized estrous cycle of non-CEH sow #503.6.

Experiment III. Evaluation of endometrial estrogen and progesterone receptors during the luteal phase of the estrous cycle of CEH and non-CEH sows.

Characteristics of Estradiol Binding to Endometrial Preparations

Figure 13 illustrates the estradiol binding curves obtained in endometrial cytosolic and nuclear preparations from CEH sows in the luteal phase of the cycle. Each point represents the mean of binding determinations from seven CEH sows. The total estradiol binding by endometrial cytosolic and nuclear preparations increased with increasing amounts of  $^3\text{H}$ -estradiol added and was represented by a curvilinear function. Moreover the specific binding of  $^3\text{H}$ -estradiol was saturable in both endometrial preparations. However, the magnitude of specific binding of estradiol in the endometrial, nuclear preparation was less than that observed in the cytosolic fraction. Nonspecific binding of  $^3\text{H}$ -estradiol for both of the endometrial preparations was linear. The nonspecific binding of  $^3\text{H}$ -estradiol was between 20-50% of the total binding to both cytosolic and nuclear fractions from the endometrium of CEH sows.

The total, specific and nonspecific binding curves of  $^3\text{H}$ -estradiol to endometrial cytoplasmic and nuclear preparations from non-CEH sows are shown in Figure 14. The mean hormone binding curves were obtained from the determination of binding characteristics of  $^3\text{H}$ -estradiol to endometrial preparations from seven non-CEH sows. The  $^3\text{H}$ -estradiol binding curves obtained in endometrial cytosolic and nuclear preparations were found to be similar. Specific binding of  $^3\text{H}$ -estradiol was saturable in endometrial, cytosolic and nuclear preparations. The percentage of total binding of  $^3\text{H}$ -estradiol which was nonspecific, was approximately 20%.

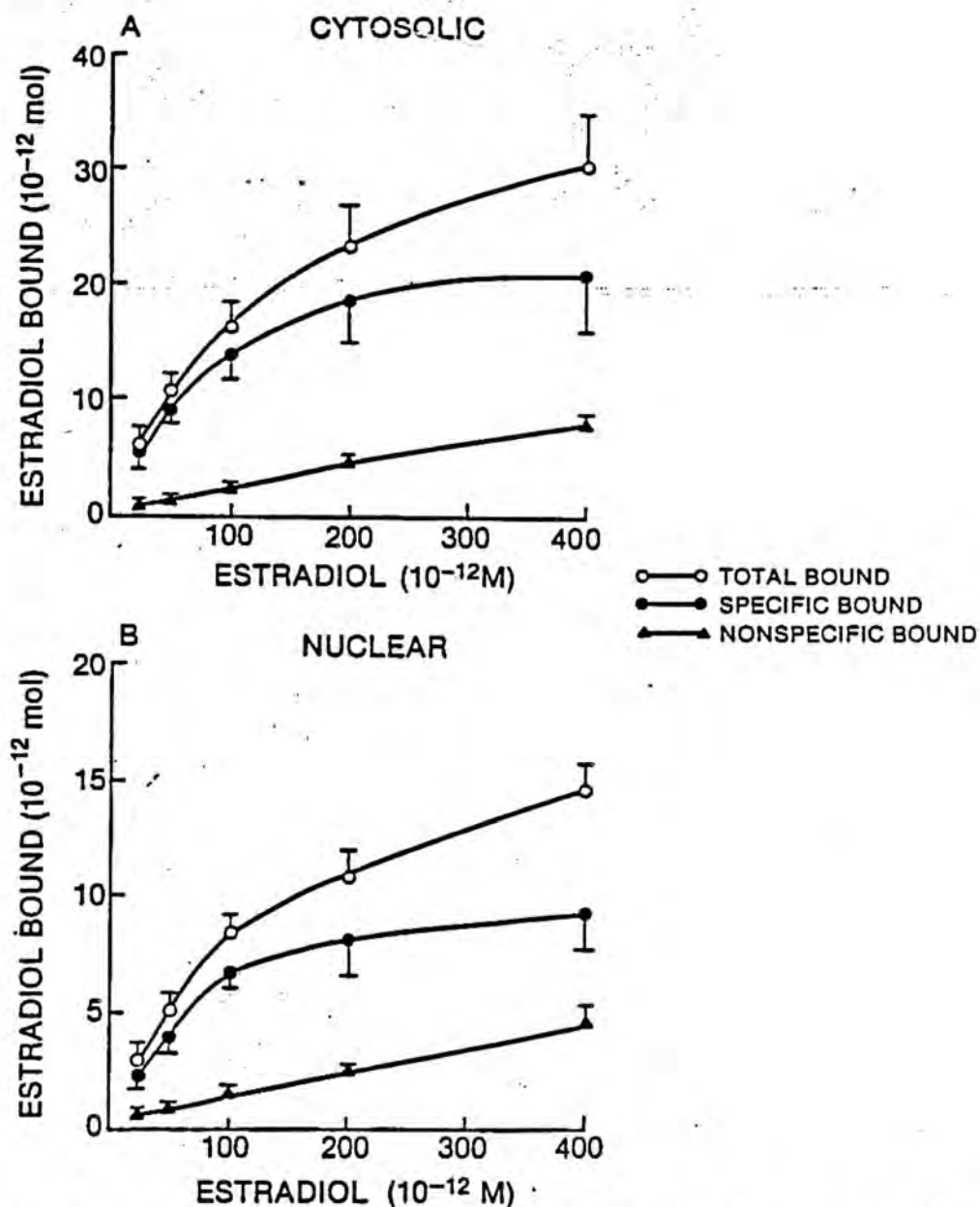


Figure 13. Characteristics of  $^3\text{H}$ -estradiol binding to endometrial preparations from CEH sows. The total, specific and non-specific binding of  $^3\text{H}$ -estradiol to the cytosolic and nuclear preparations are shown in Panels A and B. The data is expressed as the amount of  $^3\text{H}$ -estradiol bound per assay tube and each tube contained 10 and 25 mg wet weight equivalent of the cytosolic and nuclear fractions respectively, from each animal. Each point represents the mean  $\pm$  SEM of data obtained from CEH sows ( $n=7$ ).

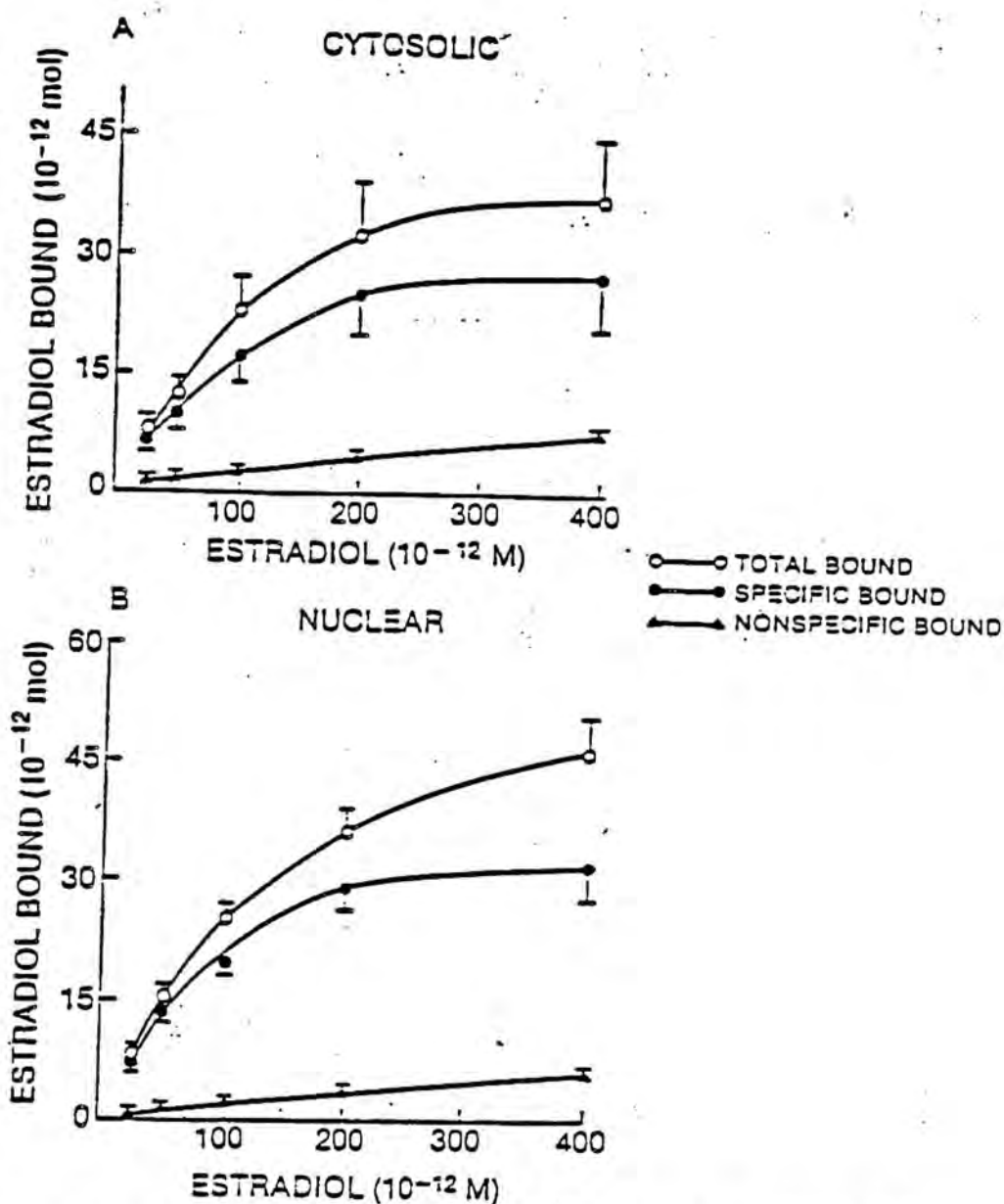


Figure 14. Characteristics of  $^3\text{H}$ -estradiol binding to endometrial preparations from non-CEH sows. The total, specific and nonspecific binding of  $^3\text{H}$ -estradiol to the cytosolic and nuclear preparations are shown in Panels A and B. The data is expressed as the amount of  $^3\text{H}$ -estradiol bound per assay tube and each tube contained 10 and 25 mg wet weight equivalent of the cytosolic and nuclear fractions respectively, from each animal. Each point represents the mean  $\pm$  SEM of data obtained from non-CEH sows ( $n=7$ ).

The mean concentrations of estradiol binding sites in endometrial, cytosolic and nuclear fractions are depicted in Figure 15. The average coefficient of determination ( $r^2$ ) obtained from linear regression analysis of Scatchard plots was  $0.95 \pm 0.01$ . There was no significant difference in the mean concentration of estrogen receptors in the cytosolic fraction of endometria from CEH and non-CEH animals ( $51.6 \pm 9.9$  and  $69.8 \pm 12.7$  fm/mg protein, respectively). The mean concentration of estrogen receptors in the nuclear fraction of endometrium of CEH sows ( $32.5 \pm 4.5$  fm/mg protein) was significantly lower ( $p < .01$ ) than that found in non-CEH sows ( $105.8 \pm 11.8$  fm/mg protein). These findings are summarized in Table 4. The average dissociation constants of estrogen receptors in the endometrial, cytosolic preparation of CEH and non-CEH sows were similar ( $0.055 \pm 0.007$  and  $0.051 \pm 0.006$  nM, respectively). Additionally the dissociation constant of estrogen receptors in the endometrial, nuclear preparation of CEH animals ( $0.064 \pm 0.003$  nM) was not significantly different from that in non-CEH animals ( $0.053 \pm 0.008$  nM).

#### Characteristics of Estradiol Binding in Individual Sows

Figures 16 and 17 show representative  $^3\text{H}$ -estradiol binding curves and Scatchard plots from endometrial, cytosolic (sow #887), and nuclear (sow #1462) preparations from two different CEH sows. The coefficient of determination ( $r^2$ ) for the estradiol binding data from sow #887 was 0.99 (Figure 16). Specific binding of estradiol to the endometrial cytosolic fraction was saturable and nonspecific binding was linear. The dissociation constant and the concentration of estradiol binding sites in the cytosolic fraction were 0.035 nM and 67.1 fm/mg protein.

In sow #1462, the total, specific and nonspecific binding curves were closer to each other than that observed in the data of other non-CEH sows (Figure 17). In general, the nonspecific binding comprised approximately 20% of the total binding of estradiol to the endometrial, nuclear preparation. However a  $r^2$  value of 0.98 was obtained from linear regression analysis of the Scatchard plot. The dissociation constant of and the number of estradiol binding sites in the nuclear preparation were 0.059 nM and 31.9 fm/mg protein.

Examples of binding data obtained from individual non-CEH sows are also presented. The characteristics of  $^3\text{H}$ -estradiol binding to endometrial, cytosolic (sow #430.2) and nuclear (sow #425.3) preparations from two non-CEH sows are presented in Figures 18 and 19. The  $r^2$  value of the binding data of sow #430.2 was 0.91 (Panel B, Figure 18). The binding affinity of  $^3\text{H}$ -estradiol to the cytosolic fraction was 0.049 nM and the concentration of estradiol binding sites was 106.9 fm/mg protein.

In sow #425.3 (Panel A, Figure 19), the characteristics of  $^3\text{H}$ -estradiol binding to the nuclear preparation were similar to that discussed previously (mean data). A coefficient of determination of 0.99 was obtained from linear regression analysis of the Scatchard plot (Panel B). The binding affinity and the concentration of binding sites in the endometrial nuclear preparation were 0.033 nM and 68.1 fm/mg protein.

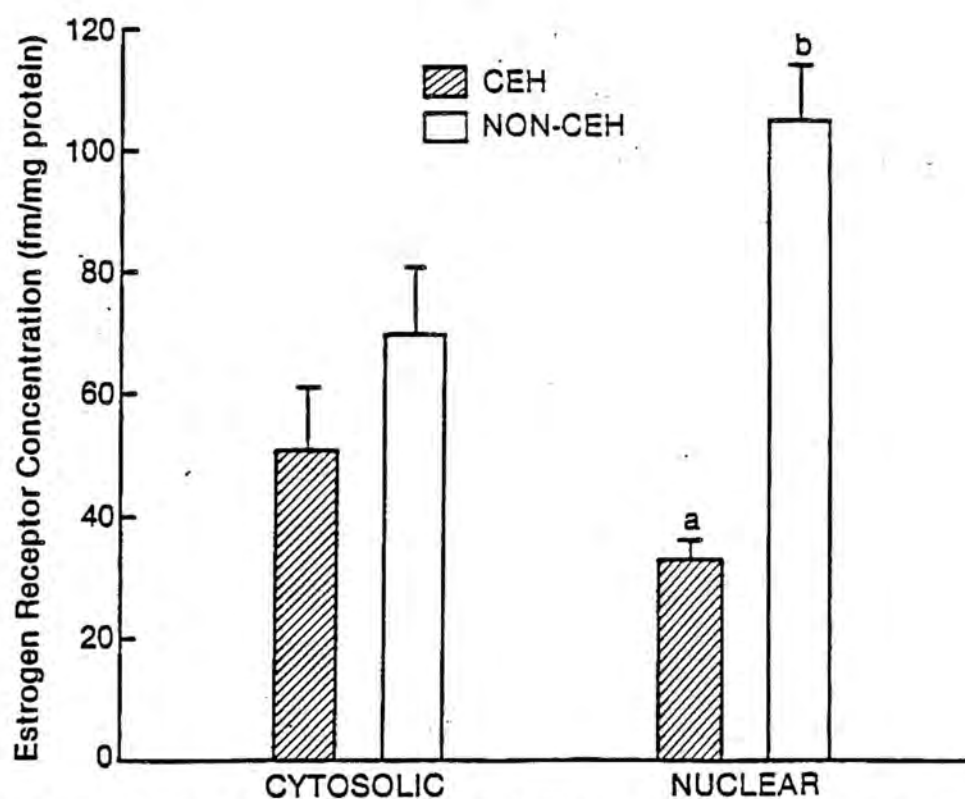


Figure 15. Comparison of the concentrations of estrogen receptors in the cytosolic and nuclear preparations of endometria from CEH and non-CEH sows. Values are expressed as mean  $\pm$  SEM. Bars with different superscripts are significantly different ( $p < .01$ ) from each other.

Animals	Estrogen Receptor Concentration (fm/mg protein)	
	<u>Cytosolic</u>	<u>Nuclear</u>
CEH	51.6 $\pm$ 9.9 <sup>a</sup>	32.5 $\pm$ 4.5 <sup>a</sup>
Non-CEH	69.8 $\pm$ 12.7 <sup>a</sup>	105.8 $\pm$ 11.8 <sup>b</sup>

Table 4. Comparison of concentrations of estrogen receptors in cytosolic and nuclear fractions of endometria from CEH (n=7) and non-CEH sows (n=7), during the luteal phase of the estrous cycle. Results are expressed as mean  $\pm$  SEM and values with different superscripts within columns are significantly different ( $p < .01$ ) from each other.

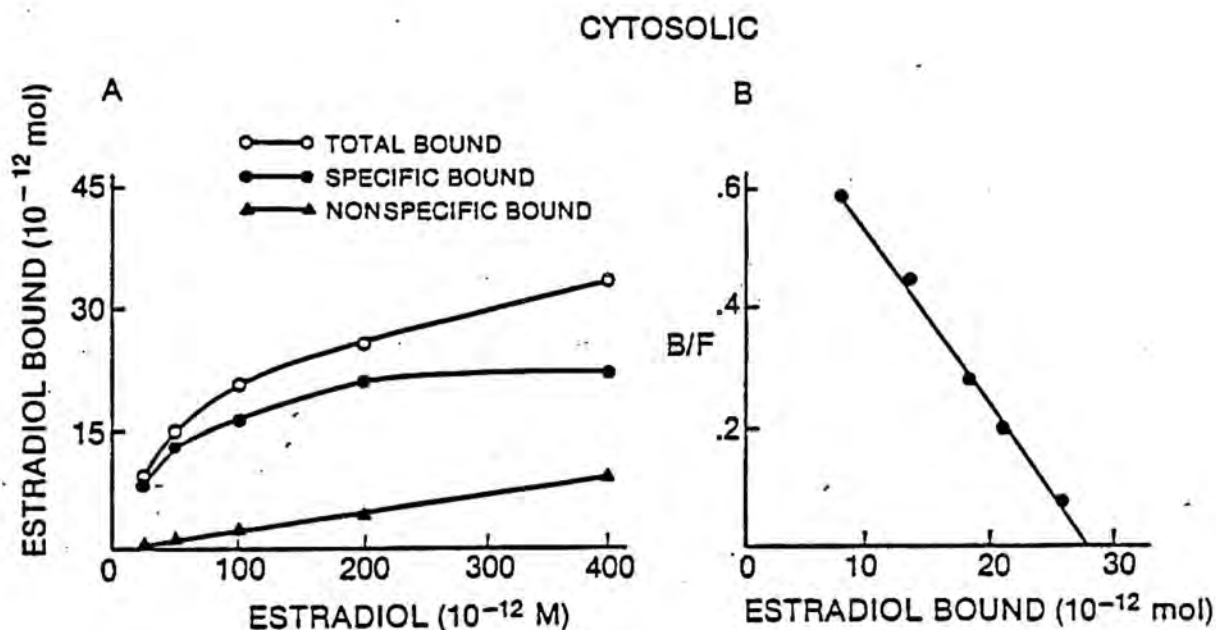


Figure 16. Binding characteristics of  $^3\text{H}$ -estradiol to the endometrial cytosolic preparation from sow #887. Panel A represents the total, specific and nonspecific binding of  $^3\text{H}$ -estradiol to the cytosolic preparation (10 mg wet weight equivalent per assay tube). Panel B shows the Scatchard analysis of the binding of  $^3\text{H}$ -estradiol to the cytosolic preparation. The dissociation constant of and the concentration of estrogen receptors in the cytosolic preparation were 0.035 nM and 67.1 fm/mg protein.

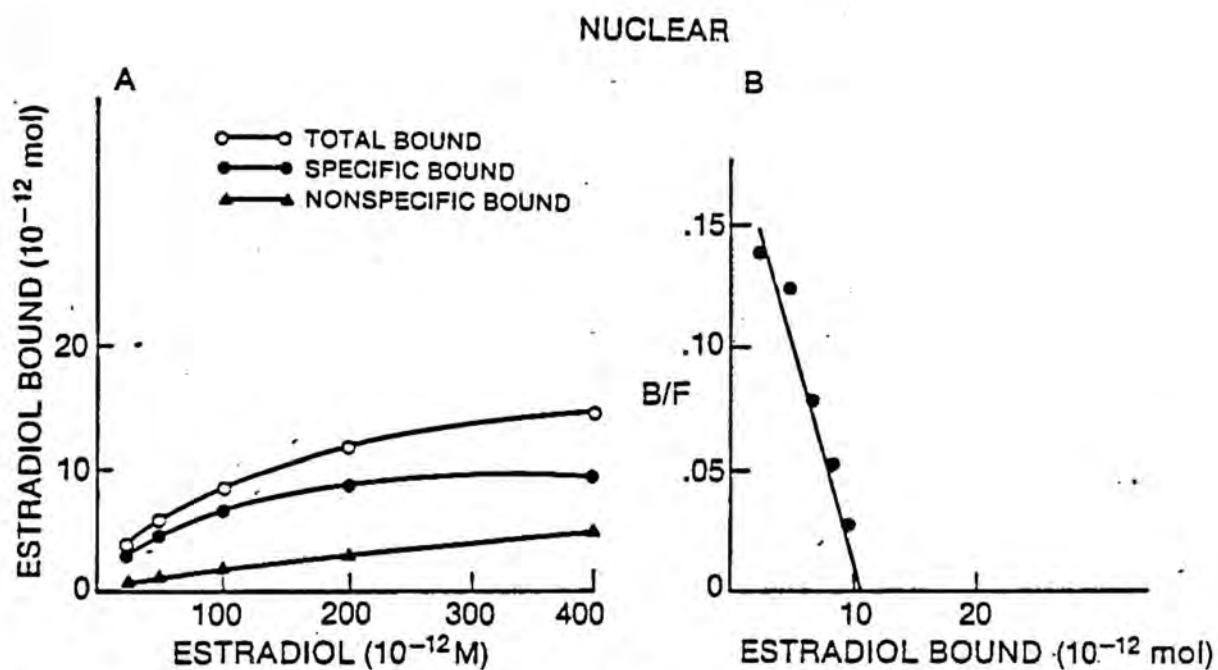


Figure 17. Binding characteristics of  $^3\text{H}$ -estradiol to the endometrial nuclear preparation from sow #1462. Panel A represents the total, specific and nonspecific binding of  $^3\text{H}$ -estradiol to the nuclear preparation (25 mg wet weight equivalent per assay tube). Panel B shows the Scatchard analysis of the binding of  $^3\text{H}$ -estradiol to the nuclear preparation. The dissociation constant of and the concentration of estrogen receptors in the nuclear preparation were 0.059 nM and 31.9 fm/mg protein.

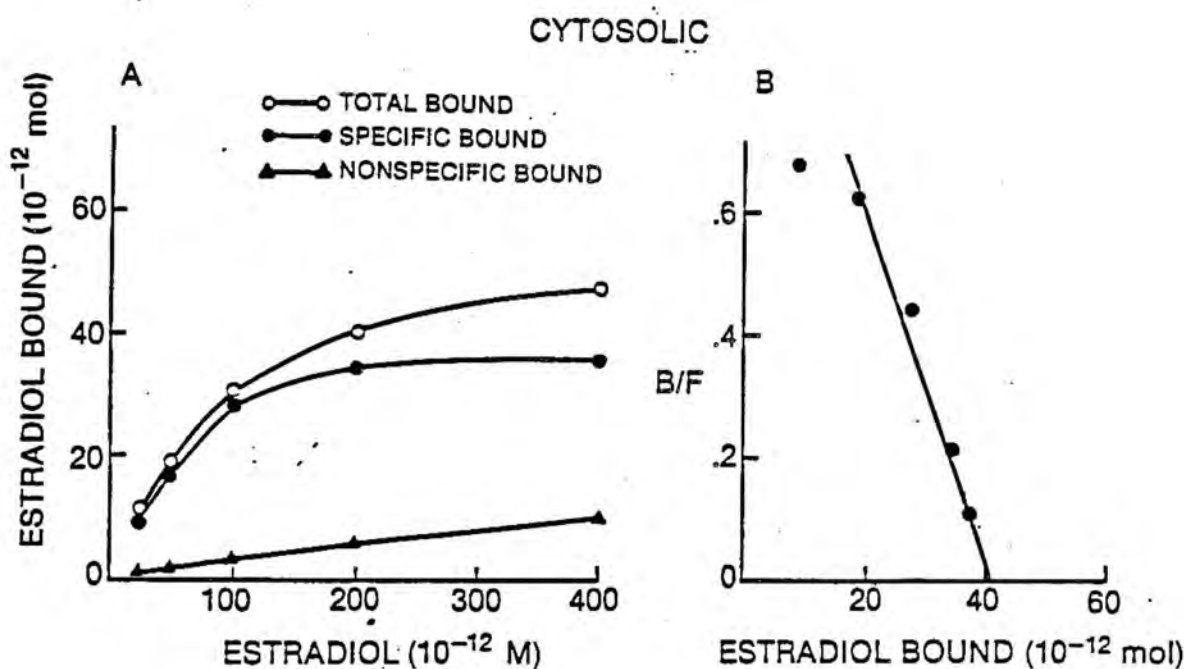


Figure 18. Binding characteristics of  $^3\text{H}$ -estradiol to the endometrial cytosolic preparation from non-CEH sow #430.2. Panel A represents the total, specific and nonspecific binding of  $^3\text{H}$ -estradiol to the cytosolic preparation (10 mg wet weight equivalent per assay tube). Panel B shows the Scatchard analysis of the binding of  $^3\text{H}$ -estradiol to the cytosolic preparation. The dissociation constant and the concentration of estrogen receptors in the cytosolic preparation were 0.049 nM and 106.9 fm/mg protein.

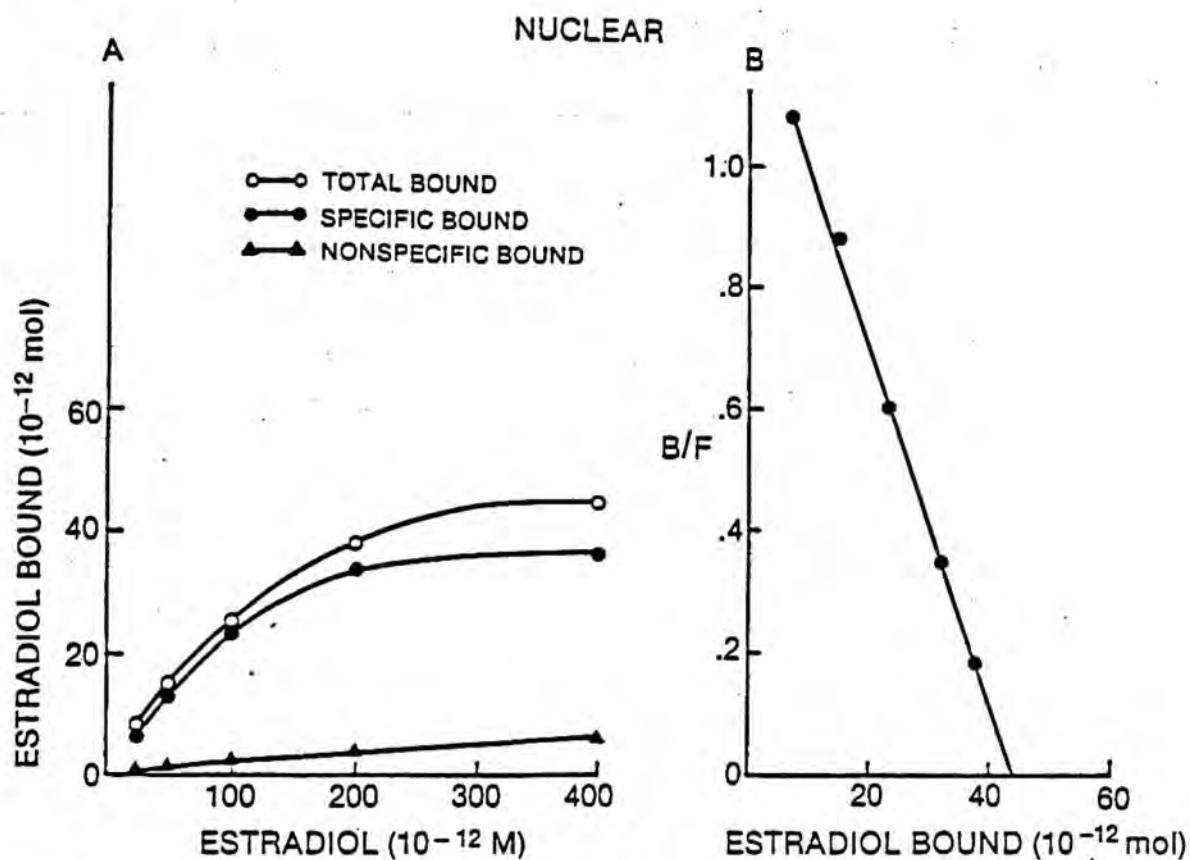


Figure 19. Binding characteristics of  $^3\text{H}$ -estradiol to the endometrial nuclear preparation from non-CEH sow #425.3. Panel A represents the total, specific and nonspecific binding of  $^3\text{H}$ -estradiol to the nuclear preparation (25 mg wet weight equivalent per assay tube). Panel B shows the Scatchard analysis of the binding of  $^3\text{H}$ -estradiol to the nuclear preparation. The dissociation constant and the concentration of nuclear estrogen receptors were 0.033 nM and 68.1 fm/mg protein.

### Quantitation of Endometrial Progesterone Receptors

The quantitation of progesterone binding sites in the endometrial cytosolic and nuclear fractions from CEH and non-CEH sows in the luteal phase was also attempted. However, there was very little specific binding of  $^3\text{H}$ -promegesterone (R5020) to both endometrial fractions (data not shown). Furthermore, linear regression analysis of Scatchard plots of  $^3\text{H}$ -promegesterone binding to these preparations usually yielded  $r^2$  values between 0.56 and 0.77. Thus cytosolic and nuclear progestin receptors could not be accurately quantitated in endometrial specimens collected from CEH and non-CEH animals during the luteal phase of the estrous cycle.

Experiment IV. Investigation of the effects of 17- $\beta$ -estradiol, progesterone and clomiphene citrate on the development of cystic endometrial hyperplasia.

Mean Level of Hormones

The mean serum concentrations of estradiol, progesterone and luteinizing hormone in ovariectomized non-CEH sows in the placebo or control group are shown in Figure 20. Each point represents the mean hormone concentrations measured in three non-CEH sows. Mean serum concentrations of luteinizing hormone remained elevated (above 5.0 ng/ml), throughout the treatment period. In contrast, the mean serum level of progesterone observed during the treatment period was below 0.5 ng/ml. During the first six weeks of the study, the mean serum level of estradiol was above 20.0 pg/ml. Fluctuations in the mean serum level of estradiol were observed during the last six weeks of the study.

The mean serum levels of estradiol, progesterone and luteinizing hormone in ovariectomized non-CEH sows with 17- $\beta$ -estradiol pellets are shown in Figure 21. Mean serum levels of progesterone and luteinizing hormone were below 1.0 ng/ml, throughout the treatment period. The mean serum level of estradiol was maintained above 160.0 pg/ml by the estradiol pellets.

In intact CEH animals with progesterone pellets, the mean serum luteinizing hormone concentrations varied between 3.0 and 5.0 ng/ml (Figure 22). After a sharp decline in the mean serum level of estradiol from weeks 2 to 4, serum estradiol concentrations varied between 25.0 and 50.0 pg/ml thereafter. Mean serum level of progesterone was observed to rise concurrently with the decline in the mean level of estradiol. The mean level of progesterone then fell from weeks 4 to 6.

For the remainder of the treatment period, the mean serum level of progesterone was maintained at approximately 6.0 ng/ml.

Figure 23 shows the mean serum levels of estradiol, progesterone and luteinizing hormone in intact CEH sows implanted with clomiphene citrate pellets. The mean serum level of progesterone remained above 5.0 ng/ml throughout the treatment period. Furthermore, a drop in the mean serum level of luteinizing hormone was observed between weeks 2 and 4. Thereafter, mean serum luteinizing hormone concentrations were below 1.0 ng/ml. After an initial fall and rise in the mean serum estradiol concentrations, a gradual decline in the estradiol level occurred from week 8 to week 12 of treatment.

The mean serum concentrations of estradiol, progesterone and luteinizing hormone observed in ovariectomized non-CEH sows in the control and estradiol treatment groups during the treatment period are listed in Table 5. There was no significant difference in the mean serum progesterone concentration between the control and estradiol treated sows ( $0.2 \pm 0.002$  and  $0.4 \pm 0.2$  ng/ml, respectively). The mean serum luteinizing hormone concentration of non-CEH sows in the estradiol pellet group ( $0.6 \pm 0.06$  ng/ml) was significantly lower ( $p < .01$ ) than that of sows in the control group ( $7.2 \pm 0.6$  ng/ml). In addition, sows in the estradiol treatment group had a significantly greater ( $p < .01$ ) mean serum estradiol concentration than control animals ( $209.0 \pm 17.7$  and  $22.1 \pm 1.6$  pg/ml, respectively).

Table 6 summarizes the mean serum concentrations of estradiol, progesterone and luteinizing hormone in intact, CEH sows in the progesterone and clomiphene citrate pellet groups. There was no significant differences in the mean serum concentrations of estradiol

and progesterone between the two groups. However, the mean serum concentration of luteinizing hormone in the clomiphene citrate treated sows ( $0.9 \pm 0.2$  ng/ml) was significantly lower ( $p < .01$ ) than that of progesterone treated sows.

#### Analysis of Pituitary Weight and Luteinizing Hormone Content

The mean weights and the luteinizing hormone content of pituitaries were also analyzed in sows from all the treatment groups. There were no significant differences in the mean pituitary weights and mean pituitary luteinizing hormone concentration in ovariectomized, non-CEH sows in the control and estradiol treated sows (Table 7). Similarly, there were no significant differences in mean weights and luteinizing hormone content of pituitaries from intact, CEH sows in the progesterone and clomiphene citrate pellet groups (Table 8).

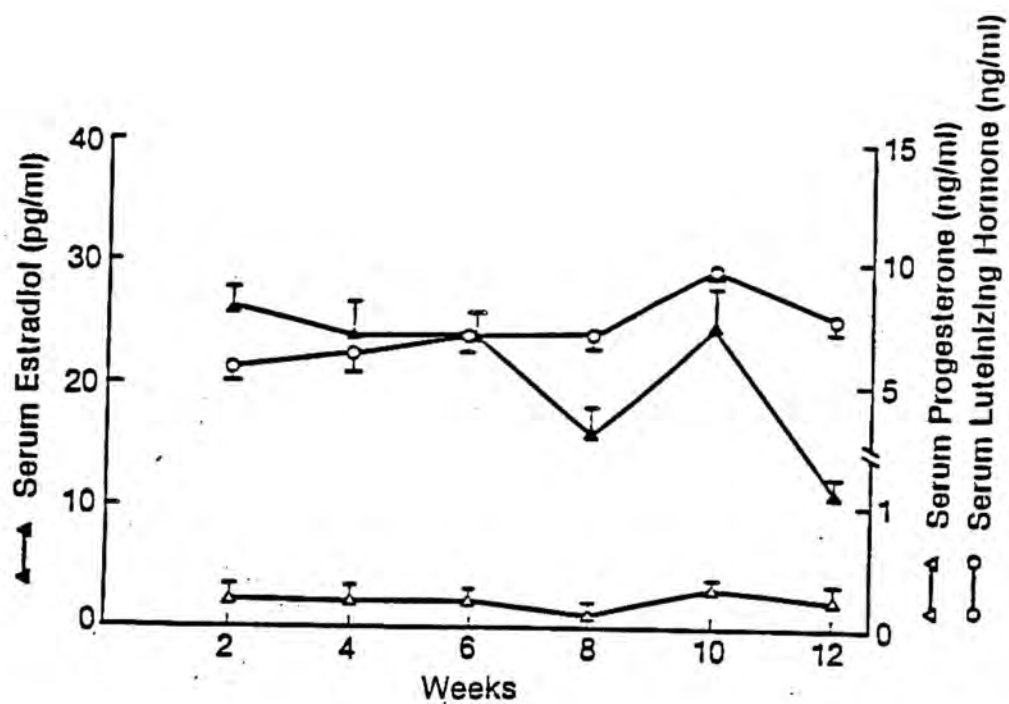


Figure 20. Serum levels of estradiol, progesterone and luteinizing hormone in ovariectomized, non-CEH sows with placebo implants. Values are expressed as mean  $\pm$  SEM. The duration of the treatment period was 12 weeks.

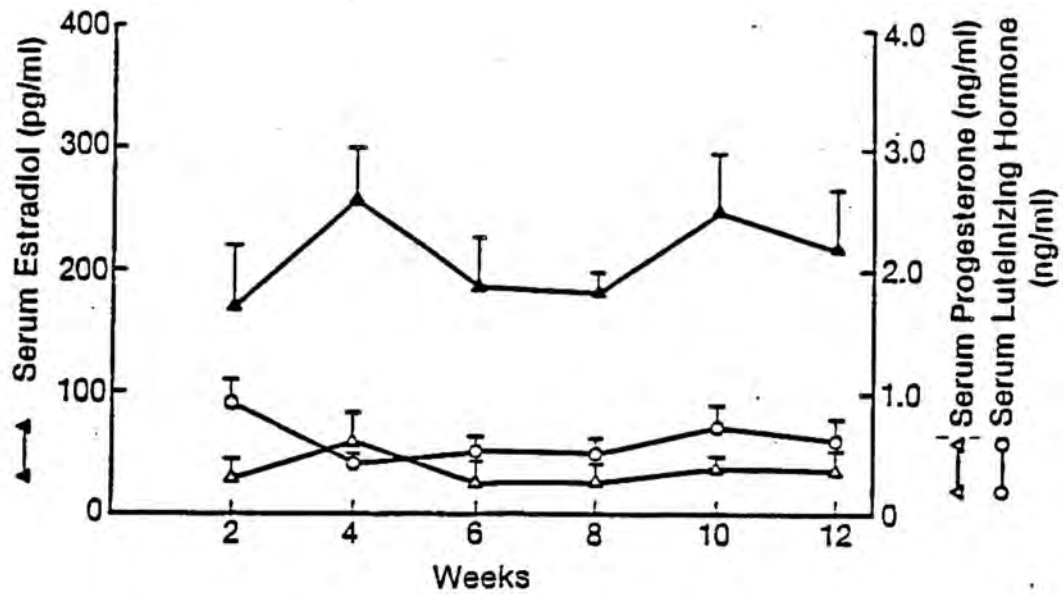


Figure 21. Serum levels of estradiol, progesterone and luteinizing hormone in ovariectomized, non-CEH sows with 17- $\beta$ -estradiol implants. Values are expressed as mean  $\pm$  SEM. The duration of the treatment period was 12 weeks.

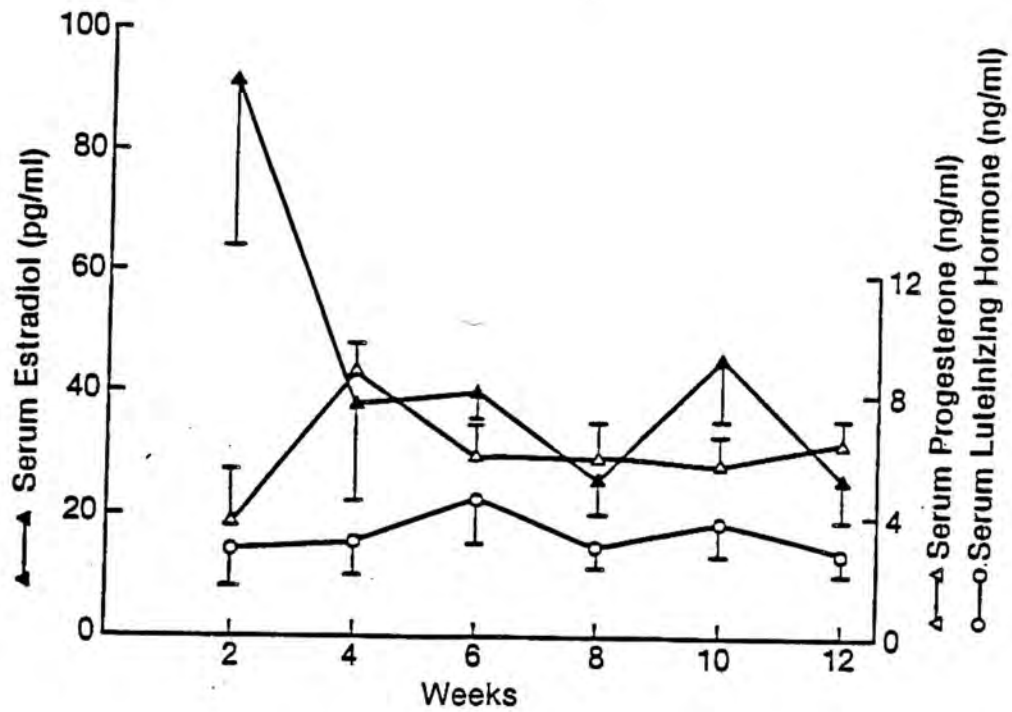


Figure 22. Serum levels of estradiol, progesterone and luteinizing hormone in intact CEH sows with progesterone implants. Values are expressed as mean  $\pm$  SEM. The duration of the treatment period was 12 weeks.

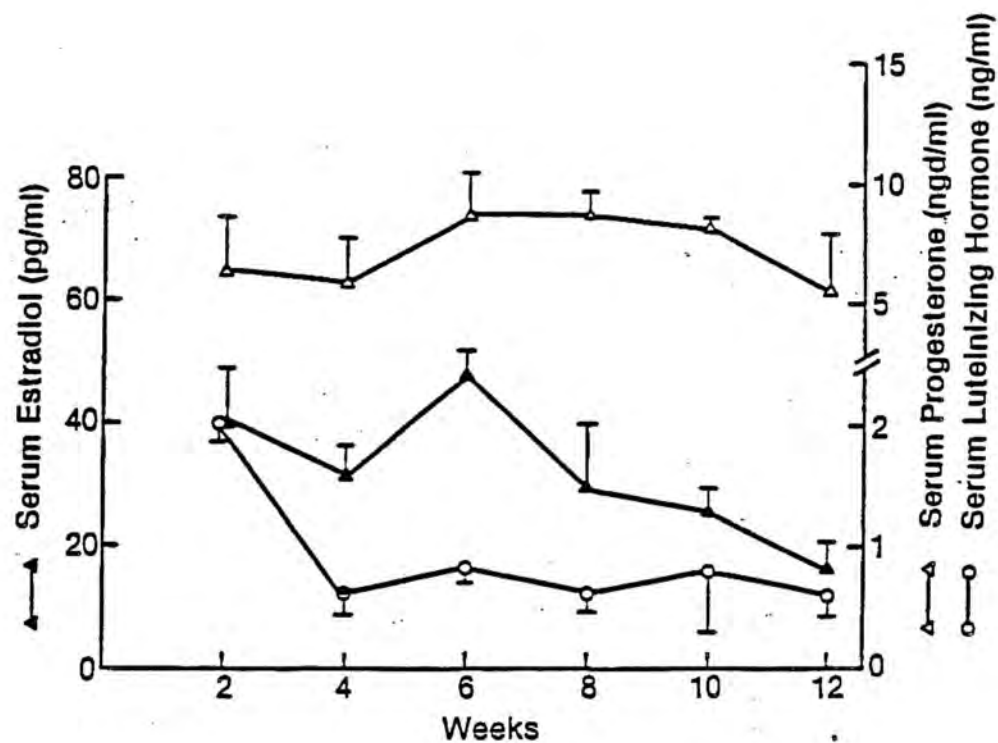


Figure 23. Serum levels of estradiol, progesterone and luteinizing hormone in intact CEH sows with clomiphene citrate implants. Values are expressed as mean  $\pm$  SEM. The duration of the treatment period was 12 weeks.

Treatment Groups	Estradiol (pg/ml)	Progesterone (ng/ml)	Luteinizing Hormone (ng/ml)
Placebo	22.1 $\pm$ 1.6 <sup>a</sup>	0.2 $\pm$ 0.02 <sup>a</sup>	7.2 $\pm$ 0.6 <sup>a</sup>
17- $\beta$ -Estradiol	209.0 $\pm$ 17.7 <sup>b</sup>	0.4 $\pm$ 0.2 <sup>a</sup>	0.6 $\pm$ 0.06 <sup>b</sup>

Table 5. Serum concentrations of estradiol, progesterone and luteinizing hormone in ovariectomized, non-CEH sows in placebo (n=3) and 17- $\beta$ -estradiol (n=4) treatment groups. Results are expressed as mean  $\pm$  SEM and values with different superscripts are significantly different ( $p < .01$ ) from each other.

Treatment Groups	Estradiol (pg/ml)	Progesterone (ng/ml)	Luteinizing Hormone (ng/ml)
Progesterone	45.4 $\pm$ 6.9 <sup>a</sup>	5.9 $\pm$ 0.5 <sup>a</sup>	3.5 $\pm$ 0.5 <sup>a</sup>
Clomiphene Citrate	31.8 $\pm$ 3.1 <sup>a</sup>	6.9 $\pm$ 0.7 <sup>a</sup>	0.9 $\pm$ 0.2 <sup>b</sup>

Table 6. Serum concentrations of estradiol, progesterone and luteinizing hormone in intact, CEH sows in progesterone (n=4) and clomiphene citrate (n=4) treatment groups. Results are expressed as mean  $\pm$  SEM and values with different superscripts are significantly different ( $p < .01$ ) from each other.

Treatment Group	Pituitary Weight ( $\mu\text{g}/100\text{ kg}$ )	Luteinizing Hormone ( $\mu\text{g}/\text{mg}$ tissue)
Placebo	670.9 $\pm$ 85.2	280.5 $\pm$ 116.4
17- $\beta$ -Estradiol	509.9 $\pm$ 115.2	112.8 $\pm$ 56.8

Table 7. The weight of and luteinizing hormone concentration in pituitaries from ovariectomized non-CEH sows in placebo (n=3) and 17- $\beta$ -estradiol (n=4) treatment groups. The pituitary weight is expressed as  $\mu\text{g}$  tissue/100 kg of body weight. The luteinizing hormone concentration is expressed as  $\mu\text{g}$  hormone/mg of pituitary tissue. Values are expressed as mean  $\pm$  SEM.

Treatment Group	Pituitary Weight ( $\mu\text{g}/100\text{ kg}$ )	Luteinizing Hormone ( $\mu\text{g}/\text{mg}$ tissue)
Progesterone	331.6 $\pm$ 29.2	317.6 $\pm$ 58.4
Clomiphene Citrate	398.3 $\pm$ 116.3	509.5 $\pm$ 121.6

Table 8. The weight of and luteinizing hormone concentration in pituitaries from intact CEH sows in progesterone (n=4) and clomiphene citrate (n=4) treatment groups. The pituitary weight is expressed as  $\mu\text{g}$  tissue/100 kg of body weight. The luteinizing hormone concentration is expressed as  $\mu\text{g}$  hormone/mg pituitary tissue. Values are expressed as mean  $\pm$  SEM.

### Gross Morphology

The appearance of the uterus and the endometrium from non-CEH and CEH sows in the various treatment groups, was photographically recorded at necropsy. The appearance of a representative uterus and endometrium from an ovariectomized non-CEH sow in the placebo group is shown in Figure 24. In non-CEH sow #416.3, the uterus was beige in color and very flaccid (Panel A). The endometrium was pink in color and smooth in texture (Panel B). However the thickness of the endometrial layer appeared to be much less than that observed in normal intact sows.

The uterus and endometrium from an ovariectomized non-CEH sow with 17- $\beta$ -estradiol implant are shown in Figure 25. The uterus from sow #1662 was very rigid, in that the uterine horns could not be easily manipulated (Panel A). The serosal layer of the uterus was light pink in color and contained some blister-like structures. The endometrium was also light pink in color and contained deep ridges, which was only observed in the endometria of sows with estradiol implants (Panel B).

Figure 26 shows the uterus and the endometrium from an intact CEH sow in the progesterone treatment group. In sow #1013, the uterine horns were very heavy and flaccid (Panel A). The outer layer of the uterus was very thin and the presence of endometrial cysts was detectable without exposing the uterine lumen. The ovaries contained a few follicles and some corpora lutea. The endometrial layer was not visible due to the presence of numerous, fluid-filled cysts (Panel B). Most of the cysts ranged between 10-20 mm in diameter. Some of the endometrial cysts were 40 mm in diameter. The fluid within the cysts was

either clear or light yellow in color. Similar characteristics were observed in the uteri and endometria from the other sows in this treatment group.

The uterus and the endometrium from a representative sow in the clomiphene citrate treatment group are shown in Figure 27. In sow #995, the uterine horns were very flaccid (Panel A). The ovaries contained few follicles. The cysts in the endometrium were flat and long (Panel B). Most of the cysts were about 40 mm in size. In general, the cysts contained a clear fluid. However, the fluid in some of the cysts was found to be gelatinous, when aspiration of the fluid from cysts was attempted.

**A****B**

Figure 24. The uterus (Panel A) and the endometrium (Panel B) from an ovariectomized, non-CEH sow (#416.3) in the placebo group.

**A****B**

Figure 25. The uterus (Panel A) and endometrium (Panel B) from an ovariectomized, non-CEH sow (#1662) in the 17- $\beta$ -estradiol treatment group.

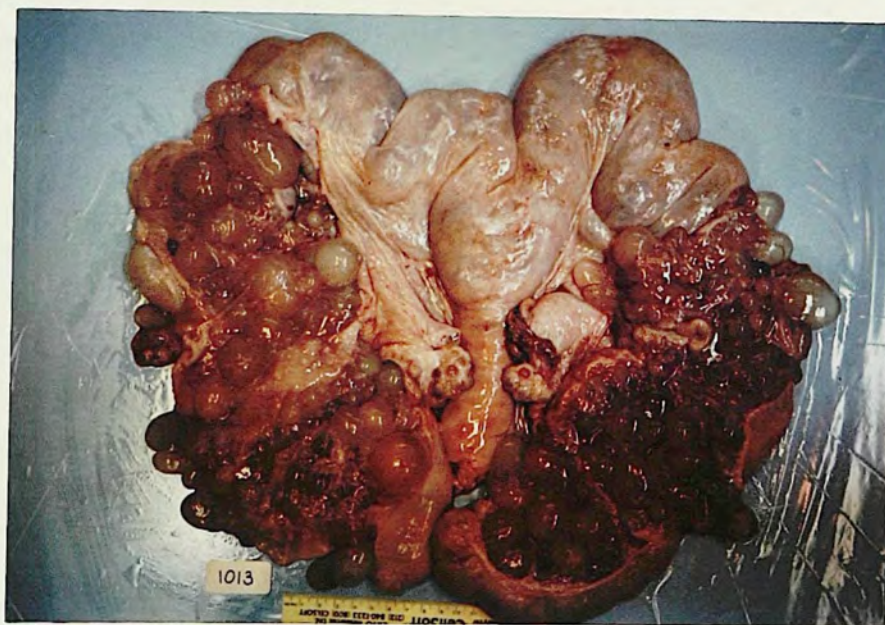
**A****B**

Figure 26. The uterus (Panel A) and endometrium (Panel B) from an intact CEH sow (#1013) in the progesterone treatment group.

A



B

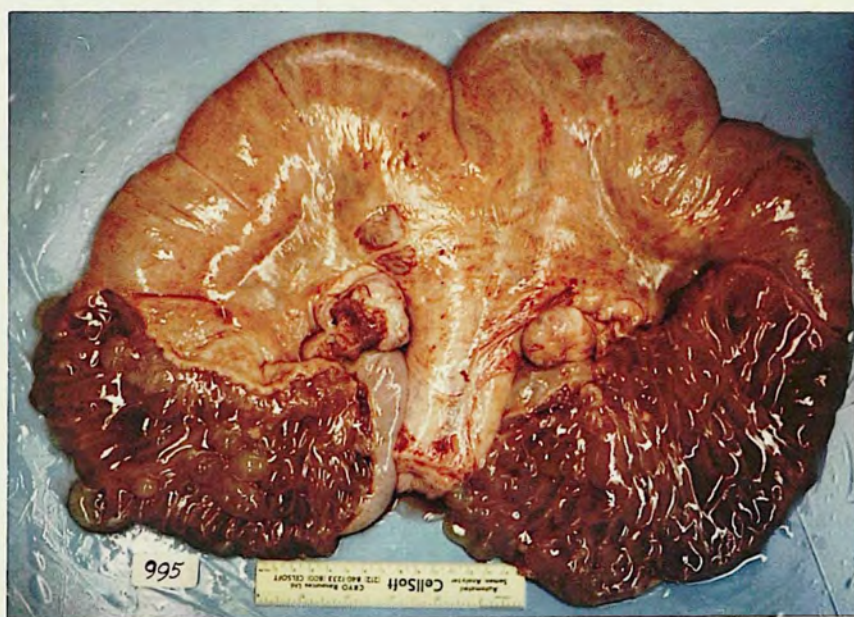


Figure 27. The uterus (Panel A) and endometrium (Panel B) from an intact CEH sow (#995) in the clomiphene citrate treatment group.

### Histological Analysis

A histological analysis of uterine tissue specimens collected from sows in each of the treatment groups was performed as previously discussed in the Material and Methods section. Figure 28 shows the histology of uterine tissue obtained from an ovariectomized, non-CEH sow in the placebo group. Panel A shows the endometrial layer, which was found to contain an intact surface epithelium. Edema was present in the endometrial stroma. Few endometrial glands were present. These glands had a flattened appearance and thus judged to be inactive (Panel B). Additionally, the boundaries of the glandular structures were diffuse. The loose organization of the endometrial stroma was evident throughout the entire layer (Figure 29). However the myometrium was normal in appearance.

Uterine tissue from an ovariectomized non-CEH sow in the 17- $\beta$ -estradiol treatment group is shown in Figure 30. The surface epithelium of the endometrium was distinct (Panel A). The stratum functionalis and the stratum basalis were distinguishable. The endometrium was slightly edematous. The endometrial stroma was hyperplastic and the stroma to gland ratio was increased. Few glands were present near the uterine lumen and the glands were dilated. More glands were located in the endometrium in the area adjacent to the myometrium (Panel B). The myometrium appeared to be normal.

The endometrial layer of the uterus from an intact CEH sow in the progesterone treatment group was edematous and very distorted (Figure 31, Panel A). The surface epithelium of the endometrium was found to be intact in some areas. Remnants of the stratum functionalis was also present. Most of the glands were flattened. The myometrium appeared to be unaffected (Panel B).

The endometrium of an uterus from an intact CEH sow treated with clomiphene citrate was completely distorted and disrupted (Figure 32). Surface epithelium of the endometrium was not detectable. However some areas of the endometrium contained a clustering of glands (Panel A). The cells of the glandular epithelium were also distorted. However the myometrium appeared to be normal (Panel B).

#### Analysis of Uterine Weights

Uterine weights of ovariectomized, non-CEH sows in the placebo and estradiol treatment groups and of intact CEH sows in progesterone and clomiphene citrate was determined at necropsy. The mean uterine weight of sows in the estradiol treatment group ( $0.75 \pm 0.3$  kg) was significantly heavier ( $p < .05$ ) than that of sows with placebo implants ( $0.14 \pm 0.05$  kg). However, the mean uterine weights of sows in the progesterone and clomiphene citrate treatment groups were similar ( $2.1 \pm 0.2$  and  $1.7 \pm 0.4$  kg, respectively).

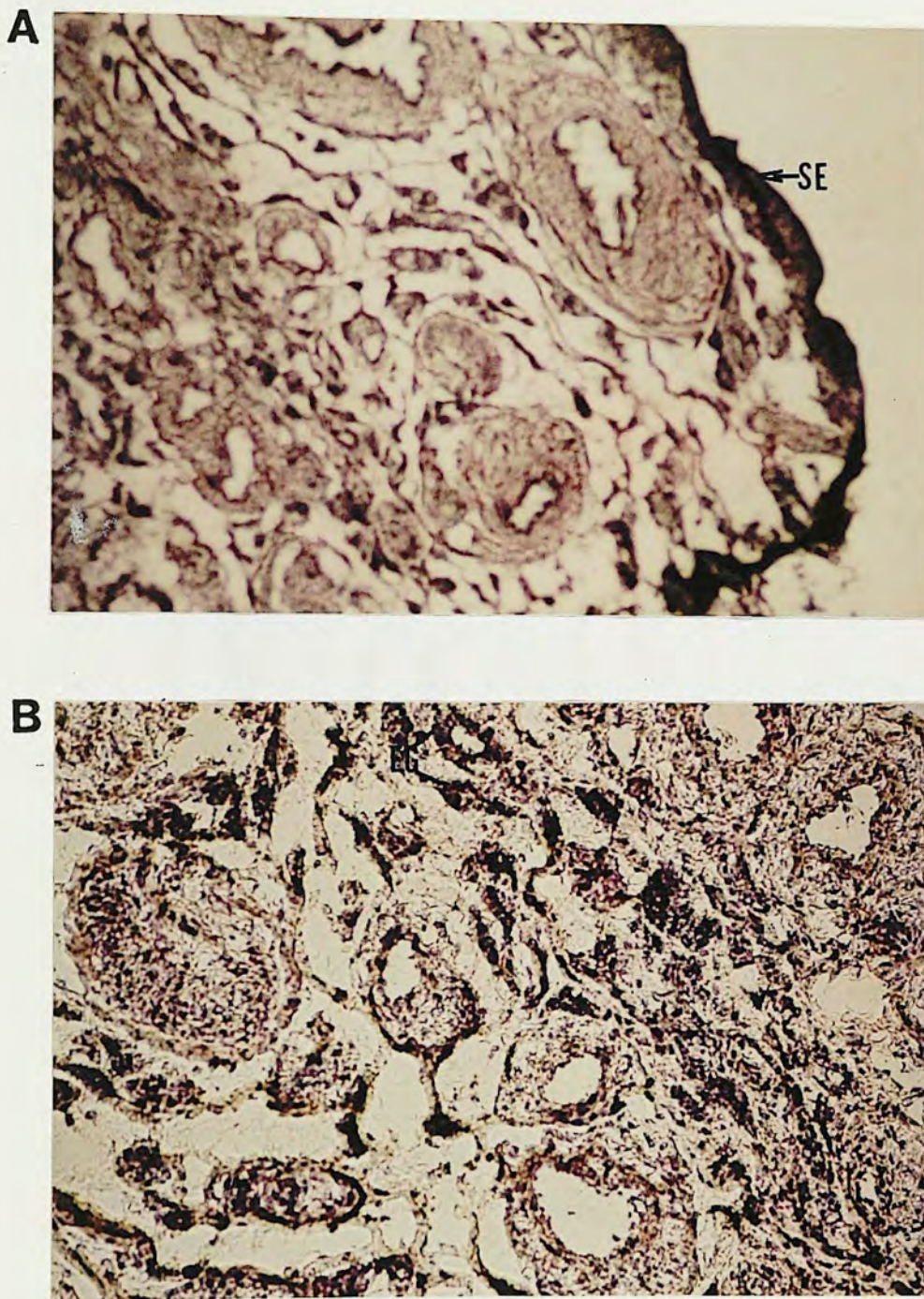


Figure 28. Histology of uterine tissue from an ovariectomized, non-CEH sow (#416.3) in the placebo group. The endometrium (Panel A, 40x) contained an intact surface epithelium (SE) but very little endometrial stroma was present. Few endometrial glands (EG) were present and they appeared to be inactive (Panel B, 100x).

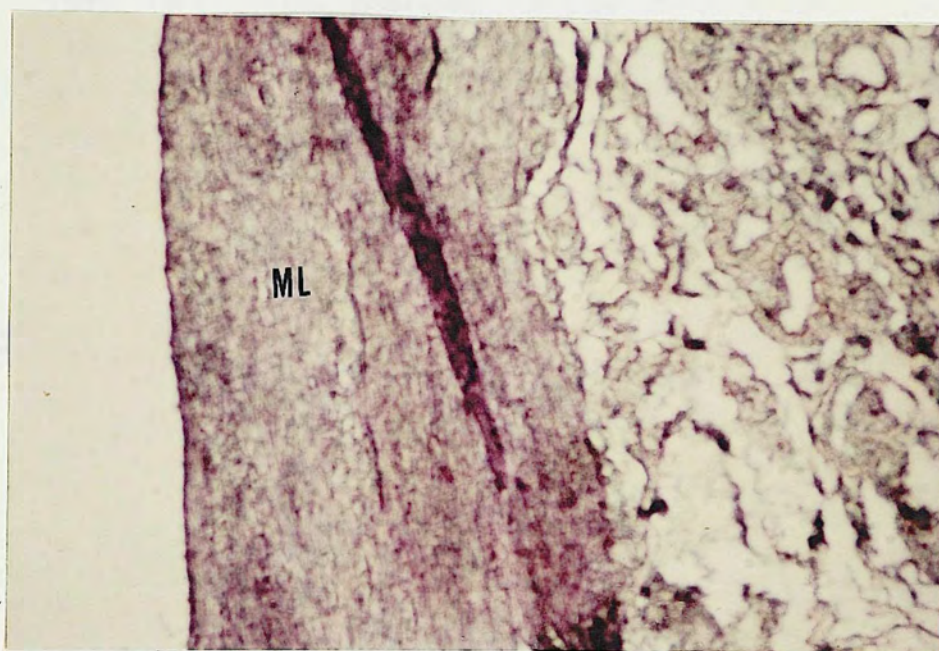


Figure 29. Histology of uterine tissue from an ovariectomized, non-CEH sow (#416.3) in the placebo group. The myometrial layer (ML) was normal in appearance (40x).

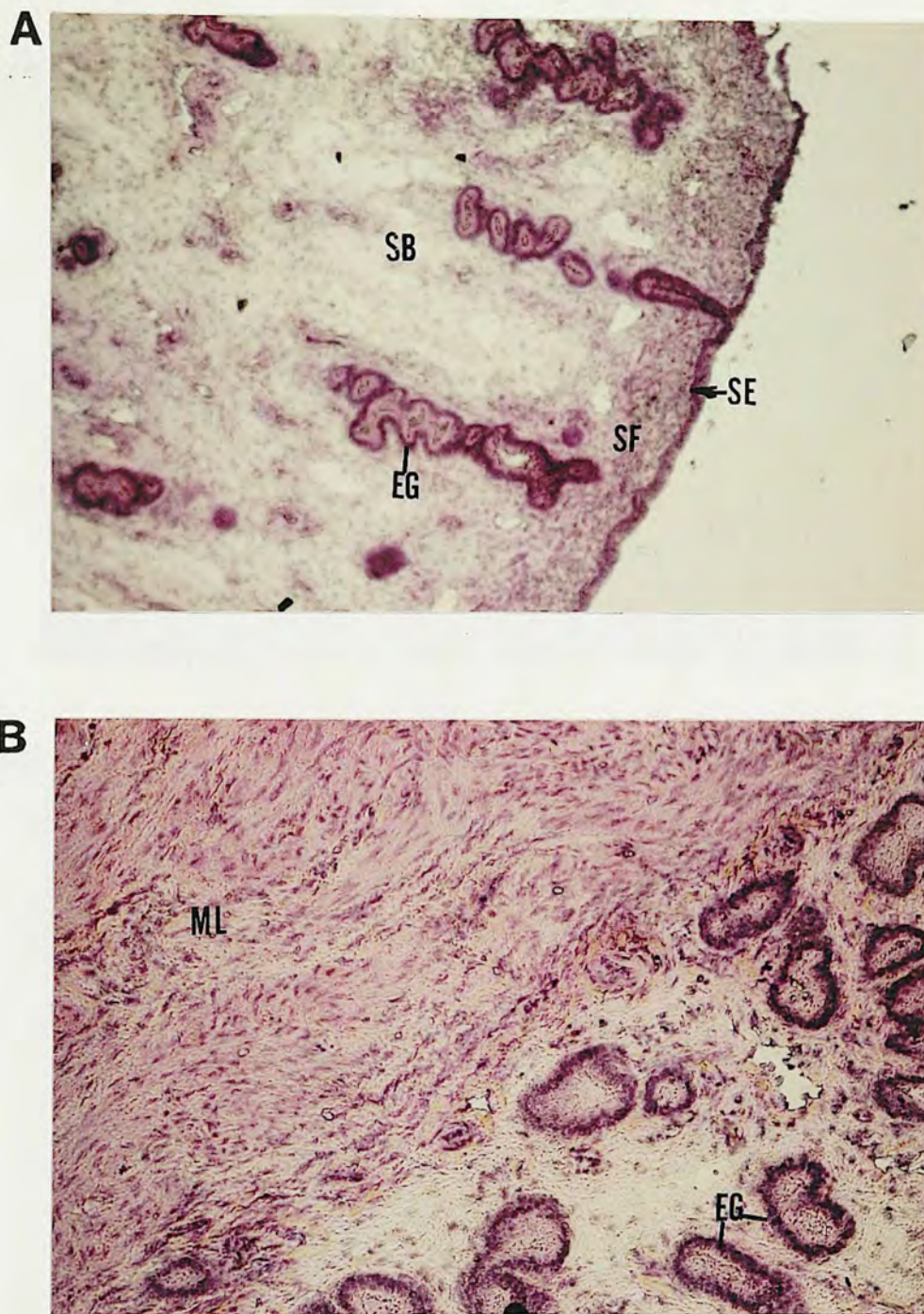


Figure 30. Histology of uterine tissue from an ovariectomized, non-CEH sow (#1723) in the 17- $\beta$ -estradiol treatment group. The endometrium (Panel A, 40x) contained an intact surface epithelium (SE). The stratum functionalis (SF) and stratum basalis were distinguishable (SB) and endometrial glands (EG) were dilated. More endometrial glands (EG) were located near the myometrial layer (ML), which was compact (Panel B, 100x).

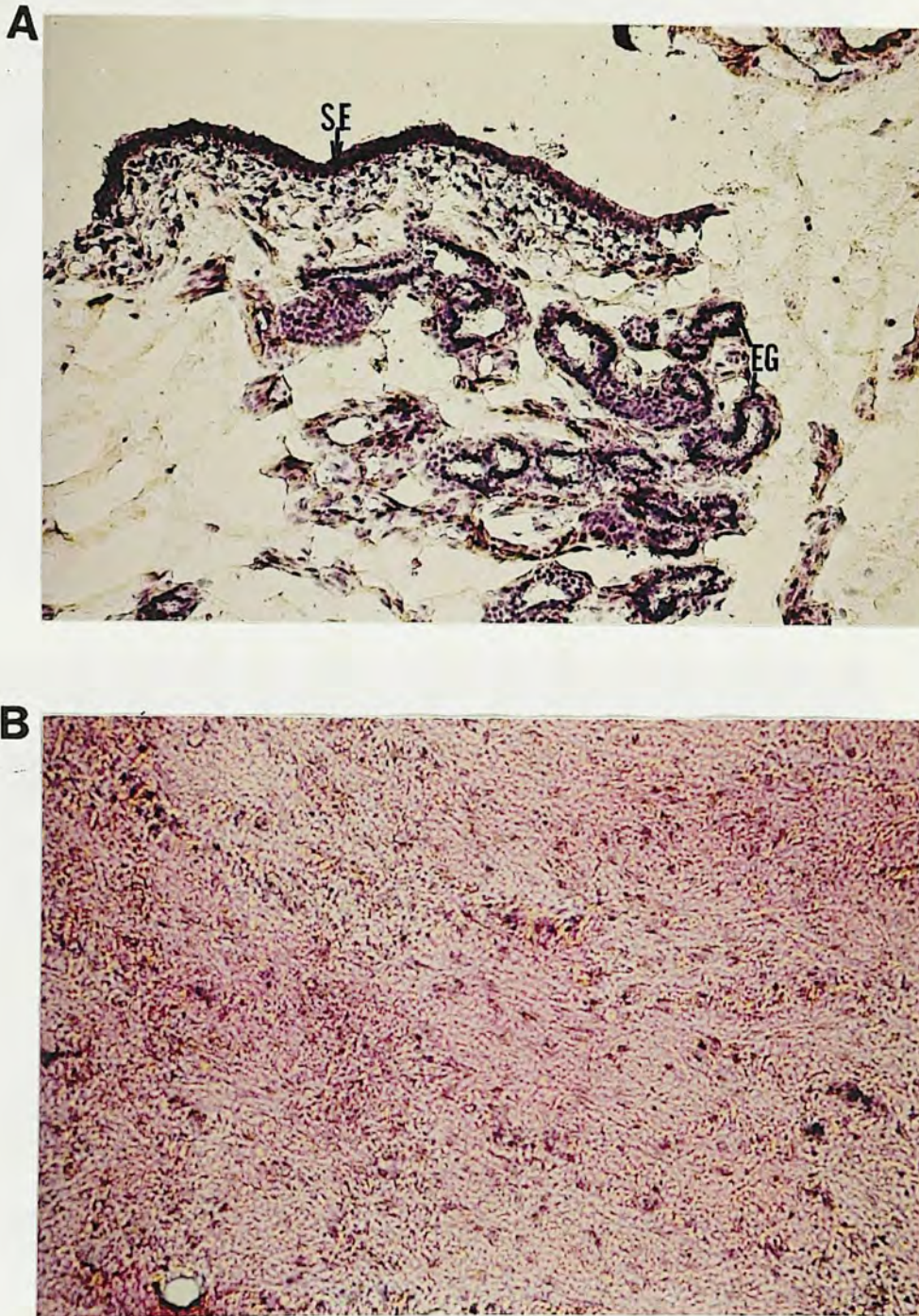


Figure 31. Histology of uterine tissue from an intact, CEH sow (#1013) in the progesterone treatment group. The surface epithelium (SE) and stratum functionalis (SF) were present in some areas of the endometrium (Panel A, 100x). Most of the endometrial glands (EG) appeared to be inactive. The myometrium was unaffected (Panel B, 100x).

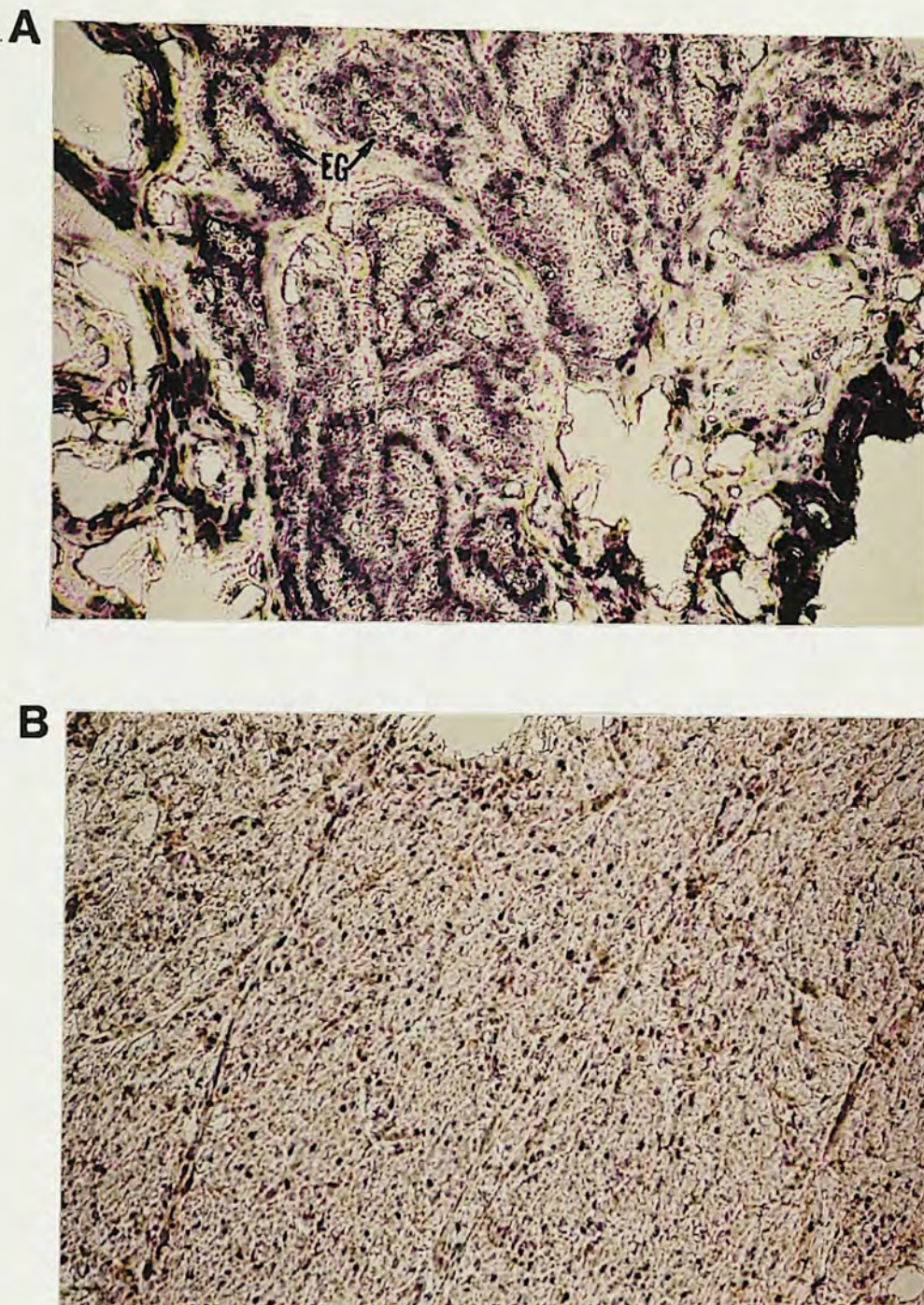


Figure 32. Histology of uterine tissue from an intact, CEH sow (#983) in the clomiphene citrate group. The endometrium was completely disrupted and distorted, however small areas containing clusters of endometrial glands (EG) were present (Panel A, 200x). The appearance of the myometrium was normal (Panel B, 100x).

### Quantitation of Endometrial Estrogen Receptors

Figure 33 shows the mean concentration of estrogen receptors measured in the cytosolic fraction of ovariectomized non-CEH sows with either placebo or estradiol implants. The ovariectomized sows in the placebo group had a significantly higher ( $p < .05$ ) mean concentration of estrogen receptors in the cytosolic preparation than sows with estradiol implants.

Ovariectomized sows in the placebo group were also found to have a significantly greater ( $p < .05$ ) mean concentration of estrogen receptors in the nuclear fraction than sows in the estradiol treatment group (Figure 34). The mean concentrations of estrogen receptors in both endometrial fractions from ovariectomized non-CEH sows with placebo or estradiol implants are summarized in Table 9.

The average concentrations of estrogen receptors in the cytosolic preparation of endometria from CEH sows in progesterone and clomiphene citrate treatment groups are shown in Figure 35. There was no significant difference in the average concentration of estrogen receptors in the endometrial cytosolic and nuclear fractions from sows in the progesterone and clomiphene citrate treatment groups. CEH sows in the clomiphene citrate treatment group had a significantly greater mean concentration of estrogen receptors in the nuclear fraction, in comparison to progesterone treated sows (Figure 36). These data are summarized in Table 10.

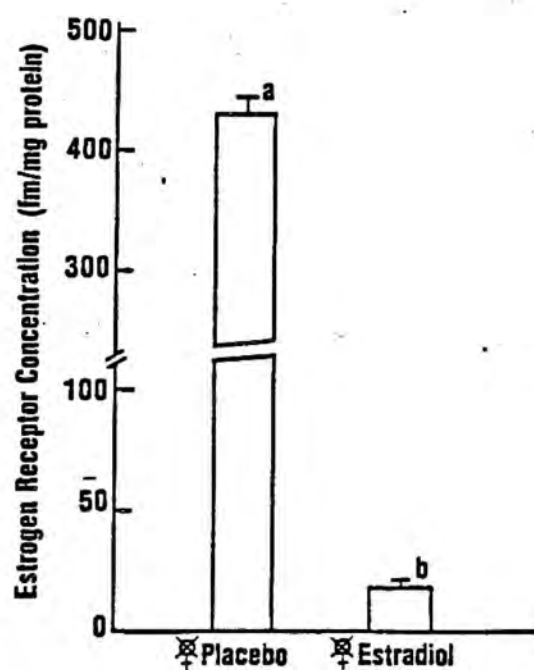


Figure 33. Concentrations of estrogen receptors in the cytosolic preparation of endometria from ovariectomized ( $\otimes$ ) non-CEH sows with placebo or 17- $\beta$ -estradiol implants. Values are expressed as mean  $\pm$  SEM. Bars with different superscripts are significantly different ( $p < .05$ ) from each other.

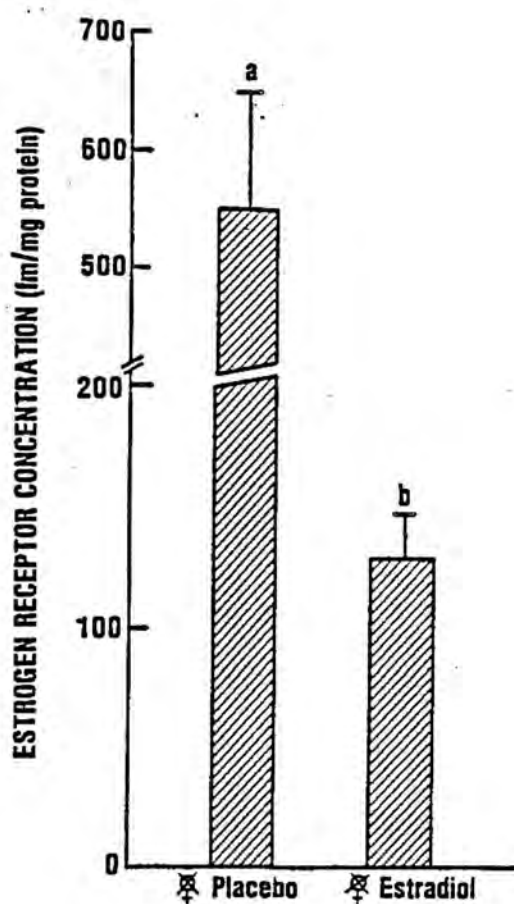


Figure 34. Concentrations of estrogen receptors in the nuclear preparation of endometria from ovariectomized (♀) non-CEH sows with placebo or 17- $\beta$ -estradiol implants. Values are expressed as mean  $\pm$  SEM. Bars with different superscripts are significantly different ( $p < .05$ ) from each other.

Treatment Groups	Estrogen Receptor Concentration (fm/mg protein)	
	Cytosolic	Nuclear
Placebo	432.5 $\pm$ 17.9 <sup>a</sup>	549.3 $\pm$ 100.3 <sup>a</sup>
17- $\beta$ -Estradiol	17.4 $\pm$ 3.7 <sup>b</sup>	129.9 $\pm$ 19.3 <sup>b</sup>

Table 9. Concentrations of estrogen receptors in the cytosolic and nuclear preparations of endometria from ovariectomized placebo (n=3) and estradiol (n=4) treated non-CEH sows. Results are expressed as mean  $\pm$  SEM and values with different superscripts within columns are significantly different ( $p < .05$ ) from each other.

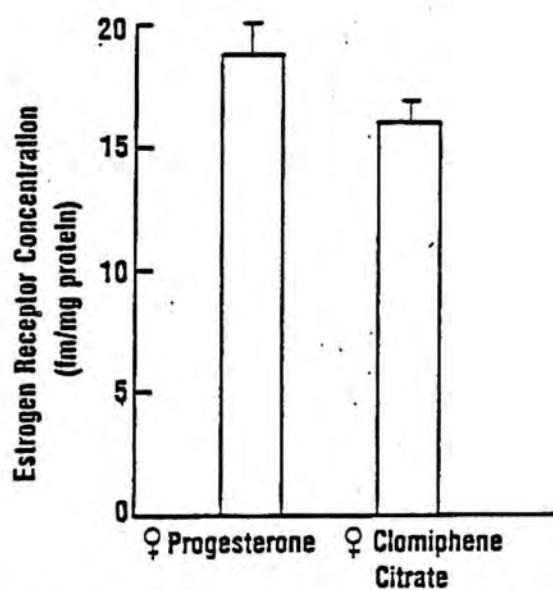


Figure 35. Concentrations of estrogen receptors in the cytosolic preparation of endometria from intact (♀) CEH sows with progesterone or clomiphene citrate implants. Values are expressed as mean  $\pm$  SEM.

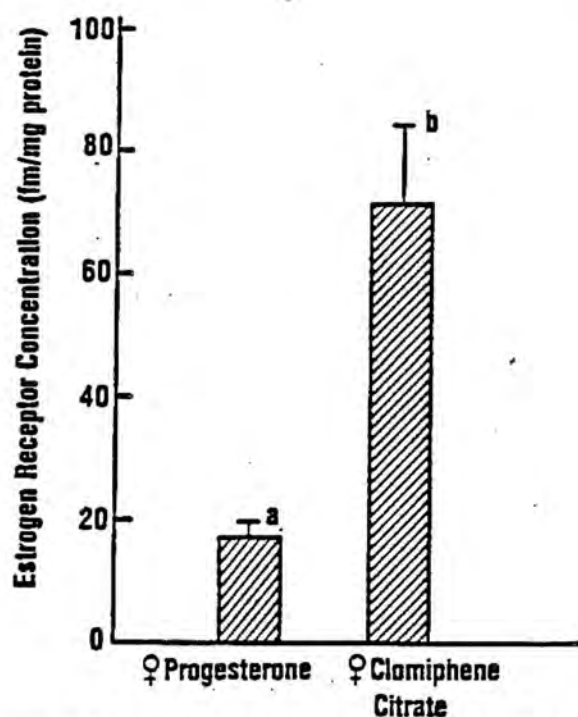


Figure 36. Concentrations of estrogen receptors in the nuclear preparation of endometria from intact (♀) CEH sows with progesterone or clomiphene citrate implants. Values are expressed as mean  $\pm$  SEM. Bars with different superscripts are significantly different ( $p < .05$ ) from each other.

Treatment Groups	Estrogen Receptor Concentration (fm/mg protein)	
	Cytosolic	Nuclear
Progesterone	18.4 ± 2.6 <sup>a</sup>	17.4 ± 2.1 <sup>a</sup>
Clomiphene Citrate	15.6 ± 1.4 <sup>a</sup>	72.2 ± 12.4 <sup>b</sup>

Table 10. Concentrations of estrogen receptors in the cytosolic and nuclear preparations of endometria from CEH sows treated with progesterone (n=4) and clomiphene citrate (n=4). Results are expressed as mean ± SEM and values with different superscripts within columns are significantly different (p<.05) from each other.

### Quantitation of Endometrial Progesterone Receptors

The cytosolic and nuclear endometrial preparations from non-CEH and CEH sows from the different treatments were assayed for the quantitation of progesterone receptors. However, the concentration of progesterone receptors could not be accurately determined in endometrial specimens from non-CEH sows in the placebo group and CEH sows under progesterone or clomiphene citrate treatment. The binding characteristics of  $^3\text{H}$ -promegesterone (R5020) to these endometrial preparations were similar to those previously discussed in the quantitation of progesterone receptors in untreated sows, during the luteal phase of the estrous cycle.

In contrast, endometrial progesterone receptors were successfully quantitated in ovariectomized, non-CEH sows treated with estradiol implants. The binding characteristics of  $^3\text{H}$ -promegesterone to the cytosolic and nuclear preparations are depicted in Figure 37. The binding curves of  $^3\text{H}$ -promegesterone were similar in the cytosolic and nuclear preparations. Each point on the binding curves represents the mean of binding data obtained from three non-CEH sows. The total promegesterone binding by endometrial cytosolic and nuclear fractions increased with the increasing amounts of  $^3\text{H}$ -promegesterone added. Specific binding of promegesterone was saturable in both preparations. A straight line was obtained for the nonspecific binding of promegesterone to both cytosolic and nuclear fractions. The nonspecific binding comprised between 20-66% of the total binding of promegesterone to both of the endometrial preparations.

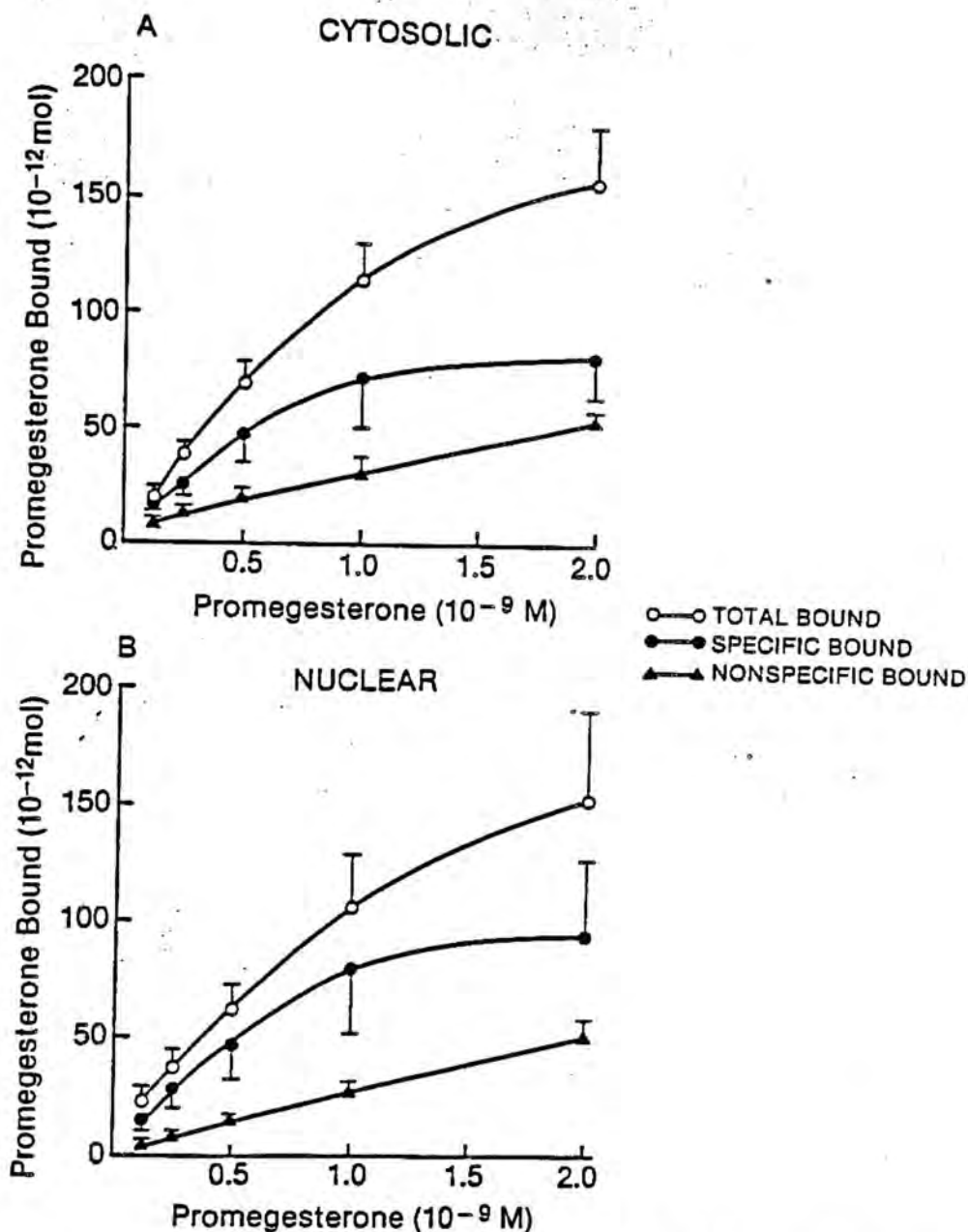


Figure 37. Characteristics of  $^3\text{H}$ -promegesterone binding to endometrial preparations from ovariectomized non-CEH sows with 17- $\beta$ -estradiol implants. The total, specific and nonspecific binding of  $^3\text{H}$ -promegesterone to the cytosolic and nuclear preparations are shown in Panels A and B. The data is expressed as the amount of  $^3\text{H}$ -estradiol bound per assay tube and each tube contained 5 and 25 mg wet weight equivalent of the cytosolic and nuclear fractions respectively, from each animal. Each point represents the mean  $\pm$  SEM of data obtained from non-CEH

The mean concentrations of endometrial progesterone receptors in the endometrial preparations from ovariectomized, non-CEH sows under estradiol treatment are depicted in Figure 38. Linear regression analysis of binding data in the cytosolic and nuclear fractions yielded average  $r^2$  values of  $0.85 \pm 0.06$  and  $0.87 \pm 0.03$ , respectively. The average concentration and dissociation constant of progestin receptors in the cytosolic fraction were  $189.2 \pm 49.3$  fm/mg protein and  $0.545 \pm 0.096$  nM, respectively. The mean concentration of progesterone receptors in the nuclear fraction was  $140.2 \pm 36.8$  fm/mg protein and the average dissociation constant was  $0.026 \pm 0.052$  nM.

#### Characteristics of Promegesterone Binding in Individual Sows

Individual profiles are presented to illustrate the type of variability present in the binding data obtained from endometrial, cytosolic and nuclear preparations. The binding curves of  $^3\text{H}$ -promegesterone (Panel A) and the Scatchard analysis (Panel B) obtained in the cytosolic preparation from non-CEH sow #1662 are shown in Figure 39. The percentage of total binding of  $^3\text{H}$ -promegesterone that was non-specific binding was approximately 40%. Linear regression analysis of the Scatchard plot yielded a  $r^2$  value of 0.96. The dissociation constant and concentration of progesterone receptors in the cytosolic fraction were 0.065 nM and 203.1 fm/mg protein.

Figure 40 contains the binding curves of  $^3\text{H}$ -promegesterone (Panel A) and the Scatchard plot (Panel B) for the nuclear preparation from non-CEH sow #419.2. The nonspecific binding of promegesterone comprised approximately 20% of the total binding. However at the highest concentration of  $^3\text{H}$ -promegesterone added (2.0 nM), the non-

specific binding was approximately 50% of the total binding. The  $r^2$  value was 0.84. The dissociation constant and concentration of progesterone receptors in the nuclear preparation were 0.025 nM and 210.8 fm/mg protein.

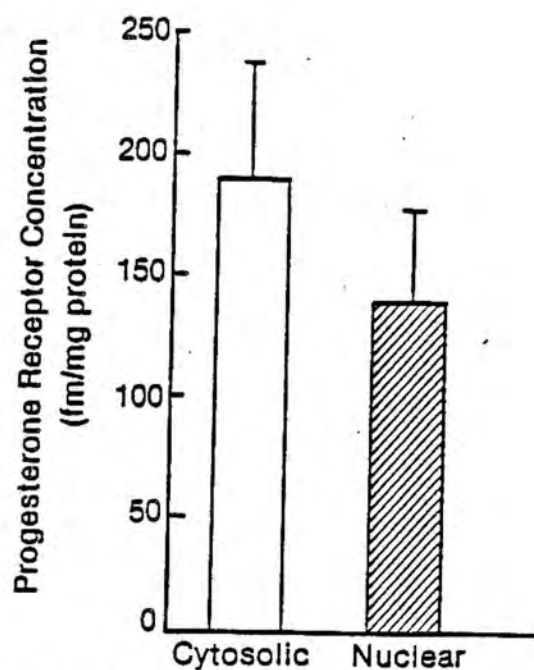


Figure 38. Concentrations of progesterone receptors in the cytosolic and nuclear preparations of endometria from ovariectomized non-CEH sows with 17- $\beta$ -estradiol implants. Values are expressed as mean  $\pm$  SEM.

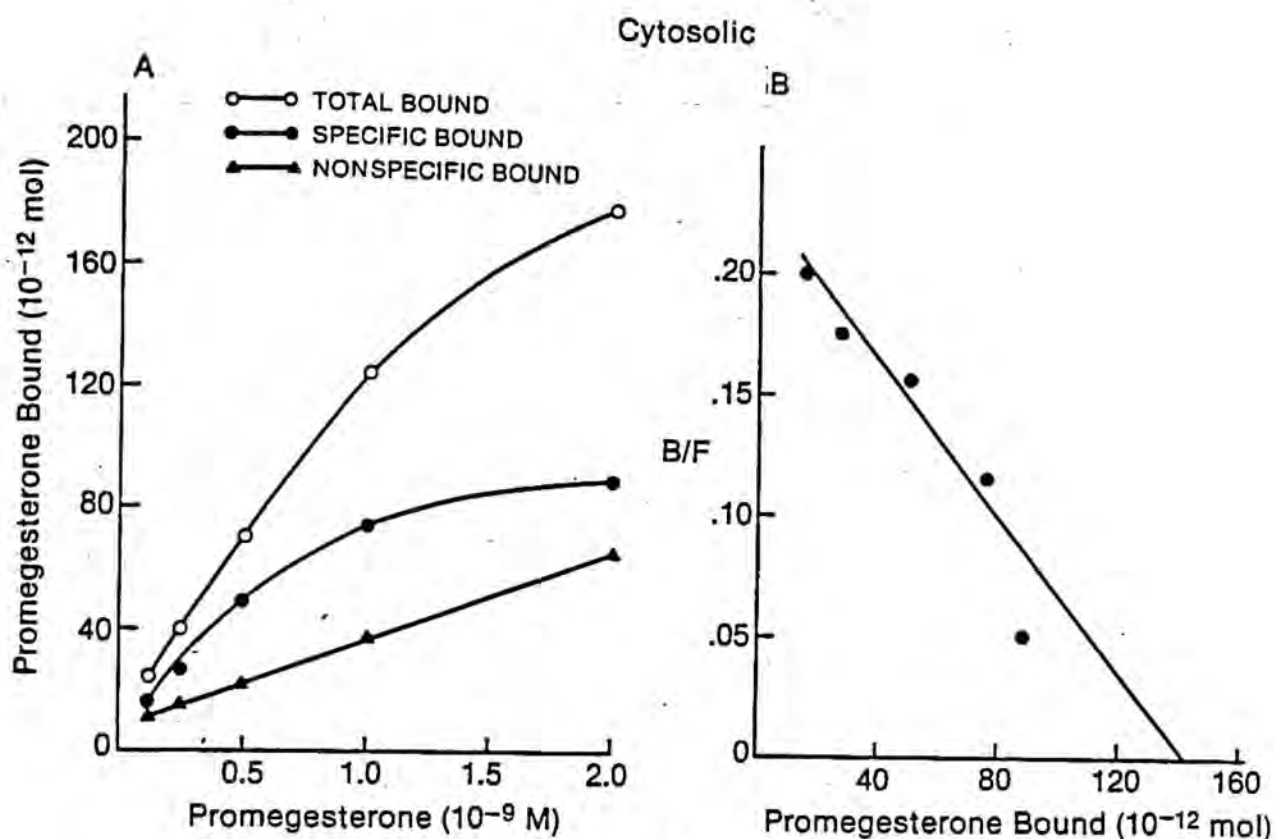


Figure 39. Binding characteristics of  $^3\text{H}$ -promegesterone to the endometrial cytosolic preparation from sow #1662. This non-CEH sow was ovariectomized and treated with  $17\text{-}\beta$ -estradiol implants. Panel A represents the total, specific and nonspecific binding of  $^3\text{H}$ -promegesterone to the cytosolic preparation (5 mg wet weight equivalent per assay tube). Panel B show the Scatchard analysis of  $^3\text{H}$ -promegesterone binding to the cytosolic preparation. The dissociation constant of and the concentration of progesterone receptors in the cytosolic preparation were 0.065 nM and 203.1 fm/mg protein.

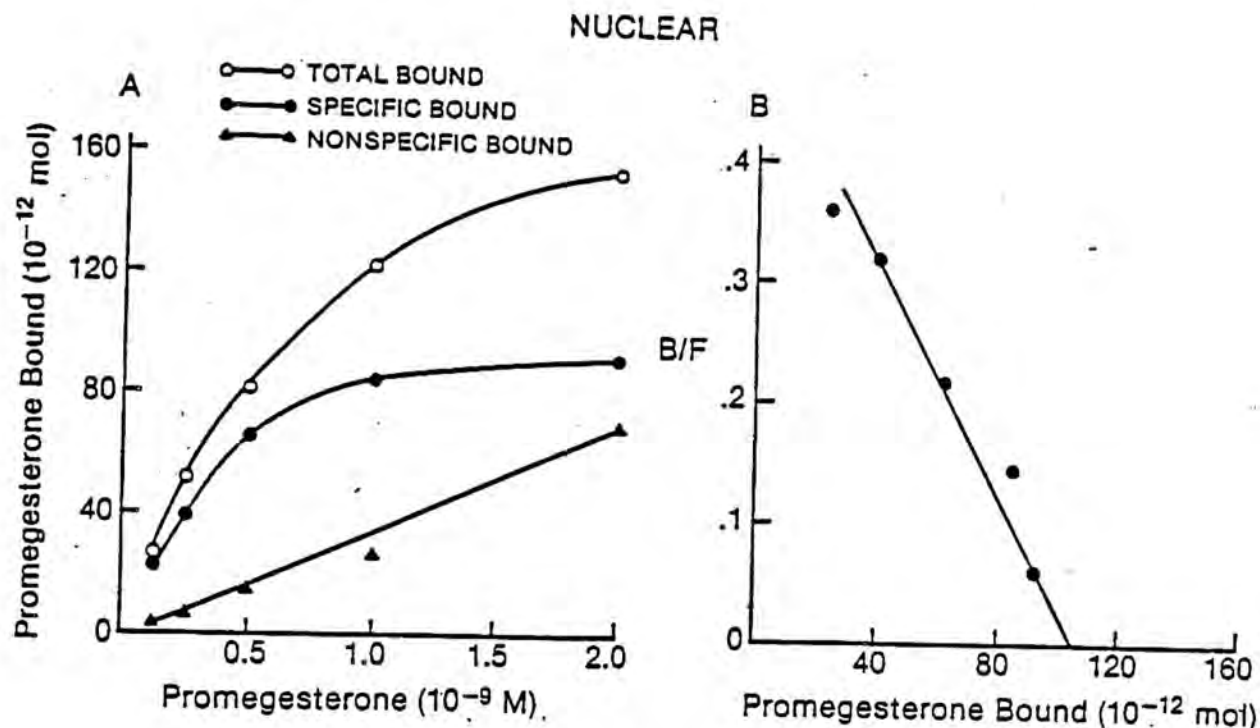


Figure 40. Binding characteristics of  $^3\text{H}$ -promegesterone to the endometrial nuclear preparation from sow #419.2. This non-CEH sow was ovariectomized and treated with 17- $\beta$ -estradiol implants. Panel A represents the total, specific and nonspecific binding of  $^3\text{H}$ -promegesterone to the nuclear preparation (25 mg wet weight equivalent per assay tube). Panel B shows the Scatchard analysis of the  $^3\text{H}$ -promegesterone binding to the nuclear preparation. The dissociation constant of and the concentration of progesterone receptors in the nuclear preparation were 0.025 nM and 210.8 fm/mg protein.

## Chapter 6

### DISCUSSION

The occurrence of cystic endometrial hyperplasia has been noted in a number of species (Jones et al., 1983), including the standard-sized pig (Thain et al., 1965; Singh et al., 1975). Thain (1965) examined the reproductive tracts from 112 non-pregnant sows and 38.2% of the tracts exhibited a hyperplastic endometrium. The endometrium of sows with CEH contained fluid-filled cysts which ranged in diameter between 0.1 and 0.3 cm. Few of the endometrial cysts were 1.0 to 3.0 cm in diameter. Similar observations were made by Singh et al. (1975) in the uterus of an infertile sow. Histological analysis of this tissue indicated an involvement of endometrial glands with this condition. Cystic dilatation and an increase in the number of glands were prominent features of the hyperplastic endometrium. However, the myometrium was found to be unaffected by the cystic changes of the endometrium. Although both groups of investigators alluded to a hormonal induction of CEH in standard-sized pigs, there was no biochemical evidence provided. Additional histological characterization of CEH in standard-sized pigs remains to be conducted.

Recently Lipetz et al. (1985) examined the histology of cystic and normal endometria from SLA miniature swine, using electron and light microscopy. This group of investigators found that the surface epithelium of the endometrium in both CEH and non-CEH sows consisted of columnar cells. Some of these cells were also ciliated. There was no difference observed in the appearance of glandular epithelium between normal and cystic endometrium. When the cells of the cyst wall were examined by electron microscopy, they were found to have a large number of mitochondria.

Additionally, the number of mitochondria in the cyst wall was greater than that found in the cells composing the glandular epithelium. It was proposed that the large population of mitochondria in the cells of the cyst wall was indicative of a high metabolic rate. This higher metabolic rate may be associated with the production and accumulation of cyst fluid. Moreover, both the higher metabolic rate and the accumulation of fluid in the cysts were proposed to be under estrogenic stimulation.

The overall appearance of the endometrium of SLA miniature pigs with CEH was similar to that reported in standard-sized pigs exhibiting this condition (Thain, 1965; Singh et al., 1975). In the present investigation we were able to document the morphology of the endometrium in the early as well as in the advanced stages of CEH. In the early stages of CEH, there were localized areas of endometrium containing cystic changes and the endometrial cysts were approximately 10 mm in diameter. During the advanced stages of CEH, the endometrium of both uterine horns contained cysts which ranged in diameter between 10 to 30 cm. Some individual cysts exceeded 50 mm in diameter. Histological examination revealed that the endometrial layer contained numerous glands during the early stages of CEH. Despite the presence of tiny cysts during the early stages of CEH, the endometrium remained intact. In general histological features of the cystic endometrium found in our study resembled those previously reported for CEH in standard-sized pigs by Singh et al. (1975). However, in the progression of CEH in SLA miniature pigs, we observed clustering of numerous endometrial glands and few of the glands also had buds which protruded into the surrounding endometrial stroma. Such changes were not reported by Singh et al. (1975). The types of changes in the endometrial glands which occur in CEH suggests that the cysts may originate from the

endometrial glands. The buds of the glands may push through the endometrial layer to eventually form cysts in the uterine lumen.

Due to the scant information available on CEH in swine, it was of interest to review the clinical literature to gain additional insight into the histopathology of endometrial hyperplasia. Several investigators have examined the different forms of endometrial hyperplasia found in women (Gusberg, 1954; Chamlain et al., 1970; Welch et al., 1977; Fox, 1984; Deligdisch et al., 1985; Kurman et al., 1985) and yet a great deal of controversy exists in the classifications of endometrial hyperplasia (Wentz, 1985). The different forms and classifications of endometrial hyperplasia were reviewed by Fox (1984). One form of endometrial hyperplasia was termed cystic glandular hyperplasia. In cystic glandular hyperplasia, small cysts in the endometrium are clearly visible. The entire endometrial layer becomes distorted to the extent where the functional and basal layers are no longer discernible. The endometrial glands vary in shape and size. Some of the glands also form buds which infiltrate the endometrial stroma. However, the myometrium remains unaffected. Since similar histological changes also occur in miniature pigs exhibiting CEH, the classification of CEH can be further specified as being cystic glandular hyperplasia. Furthermore, CEH in SLA miniature pigs has histological characteristics which are similar to those found in humans (Fox, 1984) and in standard-sized pigs (Thain, 1965; Singh et al., 1975).

Endometrial hyperplasia in women is most often associated with hyperestrogenemia (Chamlain et al., 1970; Jones et al., 1981; Jones et al., 1982) and in some animals it is due to prolonged stimulation of the endometrium by progesterone (Jones et al., 1983). The possibility of steroid

hormonal contamination of the feed was ruled out. The serum concentrations of  $E_1$ ,  $E_2$ , P, T and LH were examined throughout the synchronized estrous cycle of SLA miniature pigs, to determine if a hormonal imbalance was associated with the occurrence of CEH. The hormonal profile of the estrous cycle of the standard-sized pig has been well documented (Henricks et al, 1972; Parvizi et al., 1976; Brinkley et al., 1981; Magness et al., 1983). Similarly the hormonal profile of the estrous cycle of the SLA miniature pig was characterized by Howard et al. (1983). This latter investigation also showed that the temporal relationships between  $E_2$ , LH and P during the estrous cycle of SLA miniature pigs was similar to that reported previously for standard-sized pigs. Howard et al. (1983) observed that in SLA miniature pigs, the mean duration of estrus was  $3.1 \pm 0.2$  days and the mean length of the estrous cycle was  $22.6 \pm 0.2$  days. Furthermore, peak serum levels of estradiol were detected 24 to 48 hr prior to the preovulatory LH surge. The peak serum concentrations of estradiol was maintained for 24 hr. Serum concentrations of progesterone were observed to rise from days 3 to 10, of the estrous cycle. At the time of the initiation of this study (Howard et al., 1983), the occurrence of CEH in these animals was not known. Thus in this present investigation, the estrous cycle of SLA miniature pigs was reexamined with the animals grouped according to whether CEH was present or not. The temporal relationships between the serum levels of estradiol, LH and progesterone, during the synchronized estrous cycle of CEH and non-CEH sows, were similar to those recorded during a normal estrous cycle of SLA miniature sows by Howard et al. (1983).

In contrast to the findings of Howard et al. (1983), our investigation detected higher peak concentrations of estradiol and mean concen-

trations of progesterone during the estrous cycle of both CEH and non-CEH sows. This difference in serum concentrations of estrogen and progesterone was not due to age of the animals since the ages of non-CEH sows was similar to the ages of the animals in the previous investigation. The body weights of the animals were also similar in the two studies. On the other hand, the preovulatory LH surge detected in CEH and non-CEH sows in the present study were lower than that reported by Howard et al. (1983). The duration of the LH surge, however, was similar in both studies ( $\leq 24$  hr). These differences in the serum concentrations of  $E_2$ , P, and LH could be due to differences in assay conditions. The serum concentrations of estrone and testosterone detected during the synchronized estrous cycle of CEH and non-CEH pigs has not been previously reported.

The present examination of the mean serum concentrations of estrone and estradiol during the estrous cycle of CEH and non-CEH sows did not reveal any direct evidence of hyperestrogenemia associated with the hyperplastic condition. It should be noted that a great deal of variability was observed in the serum concentrations of estrone and estradiol in both CEH and non-CEH sows. Thus it is possible that an alteration in serum concentrations of estrogen associated with CEH may have been masked. Sows with CEH were found to have significantly lower preovulatory LH surge and serum concentrations of progesterone during the luteal phase, compared with normal sows. The significance of the lower LH surge and lower serum concentrations of progesterone in the etiology of CEH is unclear, but the striking finding of lower than normal serum progesterone concentrations in CEH sows may be an important factor in the development of CEH.

Although lower progesterone levels associated with the occurrence of endometrial hyperplasia in women has not been reported, the occurrence of a defect in the preovulatory LH surge and low serum concentrations of progesterone during the luteal phase has been observed in a group of conditions loosely defined as luteal phase defect or luteal insufficiency (Jones, 1986). A reduced level of estradiol is also sometimes found with this condition. The etiologic factors of luteal phase defect is also not clearly understood. Jones (1986) suggested that luteal phase defect may be described not only as a defect in progesterone production, but also as an endometrial pathology. Gautray et al. (1981) conducted a histological analysis of the endometrial specimens obtained from women with luteal insufficiency. Endometrial specimens were collected from the subjects on days 21, 22 or 23 of the menstrual cycle. Under normal circumstances, the highest concentrations of progesterone are found on these days. The endometrial specimens were histologically classified as pure luteal insufficiency or as luteal insufficiency with persistent estrogenic influence. In specimens of pure luteal insufficiency, the endometrial layer was very thin and contained few glands. However, most of the endometrial specimens in this study were classified under luteal insufficiency with persistent estrogenic influence. In such endometrial specimens, the glandular epithelium contained little glycogen and the glandular content of secretory products were low. The organization of the endometrial stroma was also determined to be abnormal. Moreover, the serum concentrations of progesterone were further reduced in women with luteal insufficiency with persistent estrogenic influence than in cases of pure luteal insufficiency. Serum levels of estradiol were also lower in these women, in comparison to normal women. This study provides some evidence that low

progesterone levels can be associated with persistent estrogenic influence on the endometrium, even when the levels of estradiol are also reduced.

It may be hypothesized that the endometrium of CEH sows is not exposed to "sufficient" progestational influence, due to the lower serum concentrations of progesterone. This, in turn, could also be interpreted as a predominant estrogenic influence. For instance, since there was no significant difference in the serum concentration of estrogens between CEH and non-CEH sows, presumably the endometria of these sows should be under a similar degree of exposure to estrogens. However, during the luteal phase the effects of estrogen on the endometrium of CEH sows may not be adequately opposed by progesterone and consequently there is an imbalance in the estrogen and progesterone influences on the endometrium. Perhaps the presence of this imbalance over a prolonged period of time could lead to the development of CEH.

Since hormones exert their effects on target tissues through the interaction with specific receptors, the endometrial estrogen receptors of CEH and non-CEH were also quantitated. The analysis of estrogen receptors in the endometrium of the standard-sized pig has been conducted by several investigators (Pack et al., 1978; Deaver et al., 1980; Koziorowski et al., 1984; Rexroad et al., 1984). In general, peak concentrations of nuclear estrogen receptors in the endometrium are detected near the time of estrus (Pack et al., 1976; Rexroad et al., 1984). The concentration of cytosolic estrogen receptors in the endometrium begin to rise during estrus and reach peak concentrations between days 5 and 10 of the estrous cycle. Thereafter, the concentrations of cytosolic and nuclear estrogen receptors gradually fall until day 19 of the cycle. Koziorowski et al. (1984) were able to quantitate endometrial progester-

one receptor concentrations on days 0, 13 and 17 of the estrous cycle. Peak concentrations of cytosolic progesterone receptors were detected at the time of estrus (Day 0). The lowest concentrations of endometrial progesterone receptors were detected on day 17 of the estrous cycle.

A recent investigation by Tolton et al. (1985) examined estradiol binding sites in uterine homogenates from standard-sized pigs and in contrast to earlier studies, this study detected two types of cytosolic binding sites. The mean dissociation constants of the high and low affinity estradiol binding sites were  $1.94 \pm 0.51$  nM and  $21.34 \pm 6.83$  nM, respectively. However, further data supporting the presence of two types of estradiol binding sites in porcine uterine tissue are presently unavailable. A drawback of this study was that estrogen receptors were examined in homogenates of total uterine tissue, without analyzing the endometrial and myometrial layers separately. Thus it remains to be determined if the endometrium might contain two types of estradiol binding sites, or whether the low affinity estradiol binding sites are found in the myometrium only.

Other studies which examined endometrial estrogen receptors in the standard-sized pigs have found a single class of estradiol binding sites (Pack et al., 1978; Deaver et al., 1980). Endometrial estrogen receptors in porcine uterine tissue have been reported to have a mean dissociation constant of  $0.73 \pm 0.06$  nM (Deaver et al., 1980),  $2.4 \pm 1.5$  nM (Pack et al., 1976) and  $68.7 \pm 8.1$  nM (Koziorowski et al., 1984). In SLA miniature pigs (CEH and non-CEH), the dissociation constants of estrogen receptors in the endometrial, cytosolic and nuclear preparations were similar. Our data are in close agreement with the that of Rexroad et al. (1984). These investigators found a single class of estradiol binding

sites in endometrial (cytosolic and nuclear) preparations from standard-sized pigs, with dissociation constants around 0.064 nM.

Although endometrial hyperplasia occurs in dogs, cats and horses (Jones et al., 1983), characterization and quantitation of the estrogen receptors in the hyperplastic endometrium of these species has not been conducted. Clinical information on the concentrations of estrogen receptors in the hyperplastic endometrium is also limited. Clinical investigations of the role of endometrial estrogen receptors in the etiology of endometrial hyperplasia reported that the concentration of cytosolic estrogen receptors in hyperplastic endometrium was not different from that in normal proliferative endometrium (Muechler et al., 1975; Janne et al., 1979). Both of these studies measured the concentrations of unoccupied estrogen receptors, as in the present study. In agreement with the available clinical data, there was no significant difference found in the mean concentrations of estrogen receptors in the cytosolic preparations of endometria from CEH and non-CEH sows. Gurpide et al. (1976) determined that the total (occupied and unoccupied) concentrations of nuclear estrogen receptors were similar between hyperplastic and normal proliferative endometria. The mean concentrations of unoccupied estrogen receptors was significantly lower in the nuclear preparation of endometria from CEH sows, compared with that from normal sows. Since only unoccupied, nuclear estrogen receptors were quantitated in our experiment, a direct comparison of our data with the clinical data (Gurpide et al., 1976) is not possible.

The role of the lower concentration of unoccupied, nuclear estrogen receptors associated with CEH in SLA miniature pigs is difficult to discuss based on the classical model of steroid hormone action. According to this model, free estrogen receptors are found in the cytoplasm of

cells in the target tissues (Jensen et al., 1968; O'Malley et al., 1974; Balieu et al., 1975; Chan et al., 1976). Upon entering a cell, estrogen binds to a cytoplasmic estrogen receptor and the estrogen-receptor complex undergoes a conformational change (activation). This activated form of the estrogen-receptor complex is then translocated into the nucleus. The interaction of the activated estrogen-receptor complex with nuclear binding sites result in increased RNA synthesis. The subsequent synthesis of specific proteins produce changes in target tissues. Based on this theory, unoccupied estrogen receptors would not be predominantly located in the nucleus, as only bound receptors are assumed to be translocated into the nucleus. Such an assumption is inconsistent with our results.

An alternative mechanism of estrogen action has been proposed by several investigators (Gorski et al., 1976; Sheridan et al., 1979; Gorski et al., 1984). The cytosolic estrogen receptor has been demonstrated to be only an artifact produced by the homogenization of tissue (Molinari et al., 1985). In an earlier study, Sheridan et al. (1979) presented autoradiographic data for the nuclear localization of unoccupied estrogen receptors. This study also showed that the concentration of estrogen receptors in the cytosol was related to the amount of buffer that was originally added to the uterine tissue homogenate. The addition of a large volume of buffer (40.5 ml) to the homogenate, resulted in a decrease in the concentration of nuclear, unoccupied estrogen receptors and an increase in the concentration of unoccupied estrogen receptors in the cytosolic fraction. It was suggested that homogenization and the addition of buffer disrupted a partition which normally sequesters the unoccupied estrogen receptors in the nucleus. Consequently, unoccupied estrogen receptors are able to enter into the cytosolic fraction. By means of an

indirect immunoperoxidase technique which utilized monoclonal antibodies to estrogen receptor protein, King et al. (1984) showed the nuclear localization of estrogen receptors, whether or not estrogen was present. Welshons et al. (1984) also demonstrated the nuclear localization of unoccupied estrogen receptors and concluded that unoccupied estrogen receptors are normally located in the nucleus of the intact cell.

Jordan et al. (1985) also suggested that disruption of cells during homogenization causes unoccupied receptors to be displaced into the cytosolic preparation. Furthermore, during this process, the bound estrogen receptors remain in the nucleus because of their high affinity to the nuclear binding sites. It was proposed that such events could be interpreted as translocation of bound receptors from the cytoplasm to the nucleus. However, these investigators believe that the translocation step is also an artifact. With the demonstration of the nuclear localization of unoccupied estrogen receptors by recent investigations, it appears that the classical model of estrogen action may need some modification (Schrader, 1984).

In the newer concept of the mechanism of estrogen action, estrogen is thought to enter into a cell and then into the nucleus where it binds to an estrogen receptor (Sheridan et al., 1979). Additionally, the unoccupied estrogen receptor remains in the nucleus, in the absence of, as well as in the presence of, estrogen (King et al., 1984). The binding of estradiol to the estrogen receptor induces a conformational change, but the complex remains in the nucleus (Gorski et al., 1984). Thus, the translocation step is likely an artifact of preparation. However, consistent with the classical model, the interaction of the estrogen-receptor complex with the genome of the cell produces specific changes in the target tissues.

The unoccupied estrogen receptors measured in the cytosolic preparation in our study could have conceivably been an artifact of the homogenization process. The homogenization of endometrial tissue was performed under conditions that have been demonstrated to produce artifactual estrogen receptors in the cytosolic preparation (Molinari et al., 1985). However, this matter will remain to be resolved as most of the current, simple and reliable methodology for the quantitation of estrogen receptors require the homogenization of the tissue (Utaaker et al., 1983).

A possible explanation for the lower unoccupied estrogen concentration associated with CEH in SLA miniature pigs may be proposed based on the following assumptions. First of all, unoccupied estrogen receptors are assumed to be normally located in the nucleus. Moreover, the tissue responsiveness to hormones is thought to be related to the intracellular concentration of receptors (Chan et al., 1976a). Next, based on the data of clinical investigations, if the total concentration of nuclear estrogen receptors is assumed to be similar in the endometria of both CEH and non-CEH sows, then the lower concentration of unoccupied, nuclear estrogen receptors in CEH sows could suggest a greater proportion of occupied estrogen receptors. This could indicate a greater degree of estrogenic stimulation of the endometrium of CEH sows, leading to hyperplasia. Until the methodology for the quantitation of total receptor concentrations validated for porcine uterine tissue is made available, this matter will remain unresolved.

Attempts to quantitate progesterone receptors in endometria from CEH and non-CEH sows were unsuccessful. It is thought that uterine tissue must be estrogen-primed in order to respond to progesterone stimulation (Clark et al., 1985). Specifically, in the absence of any estrogenic stimulation of the uterus, progesterone administration does not result in the formation of a secretory endometrium. Isomaa et al. (1979) have demonstrated that uterine concentrations of progesterone receptors in rabbits is under the influence of estrogen. Estradiol treatment (five days) of rabbits resulted in an increase in concentration of progesterone receptors in the cytosolic fraction of uterine tissue. In another study by Milgrom et al. (1973) ovariectomized guinea pigs receiving a single injection of estradiol were found to have a higher concentration of uterine progesterone receptors than control animals, six hr following treatment. After 24 hr, estradiol treated animals had an eight fold increase in the uterine concentration of progesterone receptors, compared to control animals. Thus, estrogen has a positive effect on uterine concentrations of progesterone receptors and thereby can influence the responsiveness of uterine tissue to progesterone. Failure to quantitate endometrial progesterone receptors in CEH and non-CEH sows may indicate that estradiol concentrations in the circulation of SLA miniature pigs, during the luteal phase of the estrous cycle, are inadequate to stimulate the production of progesterone receptors. Alternatively, a more sensitive technique of quantitating progesterone receptors may be necessary to determine the endometrial progesterone receptor concentrations in CEH and non-CEH pigs.

An in vivo study was conducted to examine whether CEH could be induced by estradiol and conversely, whether treatment with progesterone or clomiphene citrate would produce a regression of this condition. A limited amount of information is available on the long-term effects of ovariectomy on circulating concentrations of sex steroid hormones and luteinizing hormone, in the pig. Parvizi et al. (1976) reported elevated plasma concentrations of luteinizing hormone in ovariectomized, miniature pigs (Gottingen strain) 39 days post ovariectomy. Ovariectomized non-CEH sows in the placebo group were found to have a significantly higher mean concentrations of LH than the estradiol treated sows. The data of this present investigation is in agreement with Parvizi et al. (1976) even though we measured serum LH concentrations 90 days post ovariectomy. The serum concentrations of estradiol in the ovariectomized non-CEH sows in the placebo group, may reflect the synthesis of estrogen from adrenal precursors of estrogen. Short-term estradiol treatment has been demonstrated to suppress circulating levels of LH, in ovariectomized, standard-sized pigs (Lantz et al., 1972; Hoover et al., 1977). Cox et al. (1982) reported in ovariectomized, standard-sized pigs that while serum concentrations of LH were suppressed 24 hr following estradiol treatment, there was no suppression of pituitary concentration of LH. At 30 hr following estradiol treatment, serum LH concentrations began to rise and the concentrations of LH in the pituitary and gonadotropin hormone releasing hormone (GnRH) in the medial basal hypothalamus and median stalk eminence were significantly greater than in control animals. Moreover, when estradiol treatment was combined with the administration of GnRH, serum LH concentrations were higher than that observed with estradiol treatment alone. From these data it was concluded that the pituitary gland was responsive

to GnRH stimulation, even though serum LH concentrations were suppressed by estradiol. Therefore the reduction in serum LH concentrations by estradiol was partly mediated by the inhibition of GnRH release by the hypothalamus. It was proposed that in pigs, the hypothalamus is a site for the negative feedback by estradiol. Estradiol treatment has also been demonstrated to increase the LH concentration in the pituitary of intact, standard-sized pigs (Chakraborty et al., 1972). Thus, the pituitary may also normally be under the regulatory influence of estradiol. In the present study, the elevated serum LH concentrations observed in ovariectomized, non-CEH sows in the placebo group could be partly due to the release of the hypothalamus from the negative influence of estradiol from the ovaries. The level of estradiol detected in the ovariectomized non-CEH sows with placebo implants was probably a reflection of adrenal steroidogenesis. The estradiol treatment of ovariectomized, non-CEH pigs reestablished the negative feedback effect of estradiol on the hypothalamus. The serum concentrations remained suppressed since pharmacologic concentrations of serum estradiol were maintained by the estradiol pellets. However, there were no significant differences detected in the weight of and the LH content of the pituitary gland between the placebo and estradiol treatment groups, over the 90-day treatment period.

The effects of clomiphene citrate on gonadotropin secretion have been difficult to explain. Some investigators have proposed that the agonist and antagonistic properties of clomiphene citrate are attributable to the dose used (Roy et al., 1964; Boyar et al., 1970; Koch et al., 1971), while others suggest that the cis and trans isomers of clomiphene have their own distinct effects (Schally et al., 1970; Debeljuk et al., 1972). Clomiphene has been shown to suppress gonadotropins in women

(Jacobson et al., 1968; Czygan et al., 1972), rats (Greenblatt et al., 1961; Roy et al., 1964; 1964a; Schally et al., 1970) and ewes (Debeljuk et al., 1972). Hsueh et al. (1978) suggested that in rats, clomiphene citrate may regulate the release of GnRH by the hypothalamus. Furthermore, an alteration in the release of GnRH could further affect the release of LH from the pituitary. In addition, the effects of clomiphene on gonadotropin levels also differ between species (Docke et al., 1969) and clomiphene can have antiestrogenic properties in the presence of estrogen and estrogenic properties in the absence of estrogen (Czygan et al., 1972). Consequently, the mechanism by which clomiphene suppresses gonadotropin levels remains to be elucidated.

In the present study, even though serum LH concentrations were lower in sows with clomiphene citrate implants, there were no significant differences in the weight of and the LH content of the pituitary gland between clomiphene citrate and progesterone treatment groups. Thus conclusions cannot be drawn on the effects of clomiphene citrate on the pituitary glands in pigs. Moreover, serum concentrations of progesterone did not differ between the clomiphene citrate and progesterone treated sows. The similar mean serum progesterone concentrations between these two groups was partly due to the failure of the progesterone implants to maintain the desired level of progesterone. However, the endometrium of sows with the progesterone implants were probably under some progestational influence since the serum concentrations of progesterone maintained by the pellets have been previously demonstrated to maintain pregnancy in standard-sized pigs (Webel et al., 1975; Dziuk et al., 1977). The elevated level of progesterone observed in the clomiphene citrate treated sows may be due to the estrogenic properties of clomi-

phene. This is based on the finding that estradiol treatment of intact, standard-sized pigs results in the maintenance of corpus luteum function (Kidder et al., 1955; Gardner et al., 1963; Chakraborty et al., 1972). Thus the inherent estrogenic properties of clomiphene citrate can enhance luteal function and maintain elevated serum progesterone concentration in miniature pigs.

In general, estradiol treatment results in increased uterine weights (Hafez, 1980). Young et al. (1979) showed in rats that ovariectomy results in decreased uterine weight. The uterine weights of estradiol treated, ovariectomized rats were similar to intact controls. Thus in the present investigation, the greater uterine weights of estradiol treated, ovariectomized non-CEH sows was most likely due to estrogenic stimulation of uterine tissue.

Stimulation of the endometrium by estrogen and progesterone produces morphological changes throughout the reproductive cycle (Hafez, 1980; Pritchard, 1980). Moreover, prolonged estrogenic stimulation of the endometrium can lead to hyperplastic changes (Chamlain et al., 1970; Jones et al., 1981; 1982). Cystic hyperplasia of the endometrium has been observed to contain both stromal and glandular hyperplasia (Thomas, 1984). Such a condition is induced by excessive and prolonged estrogenic stimulation of the endometrium. The thickness of the endometrial stroma is compact and some areas of the stroma are aglandular. Cystic dilatation of the endometrial glands is also another characteristic of this condition. In the present study, the endometrium of ovariectomized sows in the placebo group were found to contain few endometrial glands and this was probably due to the lack of estrogenic stimulation. Furthermore estrogenic stimulation of the endometrium is necessary, before it can

respond to stimulation by progesterone (Clark et al., 1985). Thus in the absence of circulating levels of estrogen and progesterone, the endometrial glands contained little secretions. On the other hand, in sows with estradiol pellets the unopposed estrogenic stimulation of the endometrium resulted in the excessive proliferation of the endometrial stroma. The endometrial stroma was compact and the stroma to gland ratio was increased. However glands were numerous in the endometrium adjacent to the myometrial layer. Cystic dilatation of the endometrial glands was not evident. Although estradiol treatment of ovariectomized, non-CEH sows did result in endometrial hyperplasia, the histological characteristics were not similar to those found in the spontaneously occurring CEH in SLA miniature pigs.

In a similar study conducted in Fischer rats (Tang et al., 1984) regression of the endometrial glands was also observed in ovariectomized rats with placebo implants. However estradiol treatment of the ovariectomized Fischer rats resulted in the development of glandular hyperplasia. These rats exhibited glandular hyperplasia after nine mo following implantation of pellets and the serum concentrations of estradiol produced by the estradiol pellets was around 800 pg/ml. This would indicate that a significantly longer treatment period and perhaps higher concentrations of estradiol than used in the present study may be necessary to induce such changes in SLA miniature pigs. The form of CEH which arises spontaneously in SLA miniature pigs probably takes several years to develop, since young sows have not been observed to develop CEH. Furthermore, species differences may exist in the susceptibility of the endometrium to become hyperplastic under prolonged estrogenic stimulation.

The effects of progesterone and clomiphene citrate treatments on CEH were inconclusive. The endometria of both groups of sows were very distorted. This was mainly due to the presence of endometrial cysts and partly due to the presence of edema in the stroma. The presence of edema in the endometrium of sows with progesterone implants may have been due to the treatment itself. Stimulation of the endometrium by progesterone can cause edema in the stroma (Hafez, 1980). The etiology of edematous endometrial stroma in the clomiphene citrate treated sows is unknown. Lindsay et al. (1969) found that the uteri of intact ewes treated with clomiphene were abnormal. The serosal layer of the uterus was found to be very thin and this was accompanied by the accumulation of clear fluid within the uterine lumen. Such changes were detected 80 hr following an injection of clomiphene citrate. It was suggested that these changes resulted from the weak estrogenic activity of clomiphene in ewes. To our knowledge, the data on the histological effects of clomiphene citrate on porcine uterine tissue has not been reported. Thus it is not known whether the accumulation of fluid in the stroma may have been due to clomiphene citrate treatment.

In addition to the histological examination, the concentration of endometrial estrogen and progesterone receptors was also analysed in non-CEH and CEH sows in the various treatment groups. The ovariectomized, non-CEH sows in the placebo group were found to have a significantly higher concentration of estrogen receptors in both the cytosolic and nuclear preparations, compared to that in sows with estradiol implants. According to the classical model of estrogenic action, the nucleus could not contain unoccupied estrogen receptors, as previously discussed. Jungblut et al. (1978) reported high concentrations of unoccupied,

nuclear estrogen receptors 40 days post ovariectomy, in standard-sized pigs. Our data are in agreement with the findings of the study by Jungblut et al. (1978), even though our data was obtained 90 days post ovariectomy. At the present time, the findings of the histological examination cannot be correlated with the receptor data, since the effect of estradiol treatment on the total receptor concentration is unknown. However, the endometrium of the estradiol treated sows was confirmed to be under estrogenic stimulation, since progesterone receptors were successfully quantitated in the endometrial preparation from these sows. It is not known why the endometrial progesterone receptors could be quantitated only when the serum concentrations of estradiol were approximately three times the peak concentrations observed during an estrous cycle.

Due to the limited availability of animals for the in vivo experiments, it was not possible to maintain untreated, intact CEH control sows. Furthermore, in the present study the histological effects of progesterone and clomiphene citrate on CEH in SLA miniature pigs were inconclusive and thus any definitive discussion of the receptor data from the sows in these two treatment groups is not possible.

This present investigation was able to demonstrate that CEH sows had lower serum concentrations of progesterone than non-CEH sows, during the luteal phase of the estrous cycle. It should, however, be noted that CEH sows were consistently older than non-CEH sows and thus the possibility cannot be excluded that the lower progesterone level may be associated with the older age of the CEH sows. This matter could be resolved by reexamining the estrous cycle of CEH sows with age-matched non-CEH animals. Moreover, the measurement of serum concentration of sex hormone binding globulins in both type of sow may provide additional information regarding

steroid-endometrial interactions.

Other possible experiments which may be performed to obtain additional information on the etiology of CEH in SLA miniature pigs would include the measurement of both occupied and unoccupied estrogen and progesterone receptors in CEH and non-CEH sows. To accomplish this it will be necessary to validate such techniques for routine use in pigs. Furthermore, an accurate documentation of endometrial sex steroid hormone receptors in both type of sows would require the collection of tissue samples on specified days throughout the length of the estrous cycle. Such an experiment would document the nature of the cyclic variation in the concentration of endometrial sex steroid hormone receptors as well as any possible differences between CEH and non-CEH sows. The experiments conducted in the present investigation should provide useful information in designing future investigations of CEH in SLA miniature pigs, which were beyond the scope of the present study.

#### Summary and Conclusions

The present investigation was undertaken to conduct a histological and biochemical characterization of cystic endometrial hyperplasia (CEH) in the swine leukocyte antigen (SLA) strain of miniature pigs. In SLA miniature pigs, CEH appears to arise spontaneously in that there is no known chemical induction involved. In preliminary studies, cyst fluid was found to contain significantly lower concentrations of  $E_1$ ,  $E_2$ , P and T, in comparison to serum concentrations in both CEH and non-CEH sows. Electrophoretic analysis of serum and cyst fluid from CEH sows and serum and uterine flushings from non-CEH sows was also conducted. Similar electrophoretic patterns were obtained from serum samples of CEH and non-

CEH sows. However, the protein constituents of cyst fluid differed from those of serum. Furthermore, cyst fluid was found to contain a protein between the molecular weights of 31K and 45K, which was not present in uterine flushings nor in serum of either types of animals. The protein which appears in the cyst fluid does not appear to be the purple protein found in uterine fluids of standard-sized pigs.

The overall appearance of the endometrium of SLA miniature pigs with CEH was similar to that reported earlier in standard-sized pigs exhibiting this condition. In the early stages of CEH there were localized areas of the endometrium containing cystic changes and the endometrial cysts were approximately 10 mm in diameter. With the progression of this condition, the endometrium contained cysts which ranged in diameter between 10 and 30 mm, while some individual cysts exceeded 50 mm in diameter. During the early stage of the development of CEH, there was an increase in the number of endometrial glands but the endometrium and the the myometrium remained intact. With the progression of CEH there was a clustering of the endometrial glands and the glands were found to form buds which protruded into the endometrial stroma. These types of changes in the endometrial glands suggest that cysts may originate from the endometrial glands and the buds may gradually push through the endometrial layer to eventually form cysts in the uterine lumen. Furthermore, CEH in SLA miniature pigs was found to contain histological characteristics which were similar to those found in both humans and in standard-sized pigs.

The temporal relationships between the serum levels of  $E_2$ , LH and P during the synchronized estrous cycle of CEH and non-CEH sows were similar to those reported earlier during a normal estrous cycle of SLA

miniature pigs. Our findings were also similar to those reported in standard-sized pigs. Serum concentrations of  $E_1$ ,  $E_2$  and T were not significantly different between CEH and non-CEH sows, during the estrous cycle, however CEH sows were found to have significantly lower preovulatory LH surge and lower serum concentrations of progesterone than non-CEH sows, during the luteal phase of the estrous cycle. A clinical study has shown that the endometrium can be under a persistent estrogenic influence when serum progesterone levels are lower than normal during the luteal phase of the menstrual cycle. Based on this evidence, it was hypothesized that during the luteal phase of the estrous cycle, the endometrium of affected pigs was not adequately exposed to progestational stimulation and consequently this imbalance in the estrogenic and progestational influence on the endometrium over a prolonged period of time could lead to the development of CEH.

In SLA miniature pigs (CEH and non-CEH) the dissociation constants of estrogen receptors in the cytosolic and nuclear preparations were similar and the results presented here were in close agreement with those reported earlier for standard-sized pigs. The concentration of unoccupied endometrial cytosolic estrogen receptors were not significantly different between CEH and non-CEH sows and this was in concurrence with the available clinical data. The CEH sows however, were found to have a significantly lower concentration of unoccupied, nuclear estrogen receptors in the endometrium compared to non-CEH sows. A previous clinical study determined that the total concentration of nuclear estrogen receptors did not differ between normal and hyperplastic endometrium. However based on the clinical data, if the total concentration of nuclear receptors were assumed to be similar in both CEH and non-CEH sows, then the lower concen-

tration of unoccupied nuclear estrogen receptors could mean a greater proportion of the estrogen receptors were occupied in CEH pigs. This could indicate a greater estrogenic stimulation of the endometrium of CEH sows. On the other hand, the significance of the lower concentration of unoccupied nuclear estrogen receptors in the endometrium of CEH sows remains unclear, until a reliable methodology of measuring the total receptor concentration in porcine tissue becomes available.

In ovariectomized, non-CEH sows with estradiol implants, the unopposed estrogenic stimulation of the endometrium resulted in an excessive proliferation of the endometrial stroma. However, this was not accompanied by glandular hyperplasia and thus the stroma to gland ratio was increased. Cystic dilatation of the endometrial glands was also not evident. Thus, although estradiol treatment of ovariectomized, non-CEH sows did result in endometrial hyperplasia, the histological characteristics were not similar to those found in the spontaneously occurring CEH in SLA miniature pigs. In a similar study conducted in ovariectomized Fischer rats, estradiol treatment of these rats resulted in the induction of glandular hyperplasia, nine months following placement of the pellets. The estradiol pellets maintained 800 pg/ml serum concentration of estradiol. These results suggest that a longer duration of treatment and/or higher concentration of estradiol may be necessary to induce glandular hyperplasia in SLA miniature pigs. Progesterone and clomiphene citrate treatments did not appear to modify the existing hyperplastic condition over the treatment period. The endometrium of CEH sows in these treatment groups contained endometrial cysts and upon histological analysis the endometrial stroma was found to be edematous. Furthermore the endometrial layer was completely distorted due to the presence of the large

cysts. Consequently a definitive discussion on the effects of progesterone and clomiphene citrate on CEH is not possible.

The overall findings of this investigation, and comparison of data previously reported in both human and animal studies, leads to the conclusion that cystic endometrial hyperplasia in the SLA miniature pig should be classified as cystic glandular hyperplasia. This condition appears to be brought about by an imbalance in estrogenic and progestational stimulation of the endometrium over a prolonged period of time.

Appendix I. Reagents and Buffers for Sodium Dodecyl Sulfate  
Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Acrylamide Solution "A" (30% acrylamide, 0.8% Bis acrylamide)
  - a. Weigh 60 g of acrylamide.
  - b. Weigh 1.6 g of Bis acrylamide.
  - c. Add distilled water for a final volume of 200 ml.
  - d. Store at 4°C.
2. Solution "B" (0.375M Tris, 0.1% SD, 9M urea)
  - a. Weigh 45.36 g Tris.
  - b. Weigh 1 g sodium dodecyl sulfate (SDS).
  - c. Weigh 540 g urea.
  - d. Bring to 1 L with distilled water and adjust pH to 9.0.
  - e. Store at 4°C.
3. 50% Sucrose in Solution "B"
  - a. Weigh 250 g sucrose.
  - b. Dissolve sucrose in 500 ml of Solution "B"
4. Solution "C" (0.12M Tris, 0.1% SDS)
  - a. Weigh 14.52 g Tris.
  - b. Weigh 1 g SDS.
  - c. Bring to 1 L with distilled water and adjust pH to 6.8.
  - d. Store at 4°C.
5. 10x Electrode Buffer (0.2M Tris, 1.92M glycine, 1% SDS)
  - a. Weigh 48.4 g Tris.
  - b. Weigh 288.4 g glycine.
  - c. Weigh 20 g SDS.
  - d. Bring to 2 L with distilled water.
  - e. Adjust pH to 8.3.
6. Coomassie Blue Solution (35% methanol, 10% acetic acid, 0.07% coomassie brilliant blue)
  - a. Measure 175 ml methanol.
  - b. Combine 0.35 ml coomassie brilliant blue in methanol.
  - c. Add 50 ml acetic acid.
  - d. Stir for 10 min.
  - e. Bring to 500 ml with distilled water and stir an additional 15 min.
  - f. Filter staining solution through two layers of Whatman #1 paper.

## Appendix II. Reagents and Buffers for Radioimmunoassays (RIA)

### Steroid Hormone RIA

#### 1. 0.5M Sodium Phosphate (monobasic)

- a. Weigh 69.005 g  $\text{NaH}_2\text{PO}_4$  into a 1 L volumetric flask and add approximately 500 ml of distilled water.
- b. Place volumetric flask on a magnetic stirrer, until the sodium phosphate is completely dissolved.
- c. Add distilled water to the sodium phosphate solution for a final volume of 1 L.
- d. Store in 1 L bottle at 0-4°C.

#### 2. 0.5M Sodium Phosphate (dibasic)

- a. Weigh 70.98 g  $\text{Na}_2\text{HPO}_4$  into a 1 L volumetric flask and add approximately 500 ml of luke warm distilled water.
- b. Place volumetric flask on a magnetic stirrer, until the sodium phosphate is completely dissolved.
- c. When the solution is at room temperature, add distilled water for a final volume of 1 L.
- d. Store in 1 L bottle at 0-4°C.

#### 3. PBS (0.14M NaCl, 0.01M $\text{NaPO}_4$ , 1:10,000 merthiolate)

- a. Weigh 145 g NaCl in 1 L beaker. Dissolve in approximately 500 ml distilled water.
- b. Add 1.75 g merthiolate.
- c. Add 120 ml 0.5M  $\text{NaH}_2\text{PO}_4$ .
- d. Add 240 ml 0.5M  $\text{Na}_2\text{HPO}_4$ .
- e. Dissolve completely and transfer to large storage bottle.
- f. Dilute to 17.5 L with distilled water.
- g. Adjust pH to 7.0 and store at 0-4°C.

#### 4. 0.1% PBS-Gelatin (PBS-G)

- a. Place 999 ml PBS in a beaker on a magnetic stirrer.
- b. Add 1 g gelatin while stirring.
- c. Heat to 40°C to dissolve gelatin.
- d. Adjust pH to 7.1 and store at 4°C.

#### 5. Dextran-Charcoal Suspension (0.5% charcoal, 0.05% dextran)

- a. Add 5 g of charcoal to storage bottle containing stirring bar.
- b. Weigh 0.5 g of T-70 dextran and add to 1 L of 0.1% PBS-G, while stirring.
- c. Transfer dextran and PBS-G mixture to storage bottle containing charcoal and mix thoroughly.
- d. Store at 4°C.

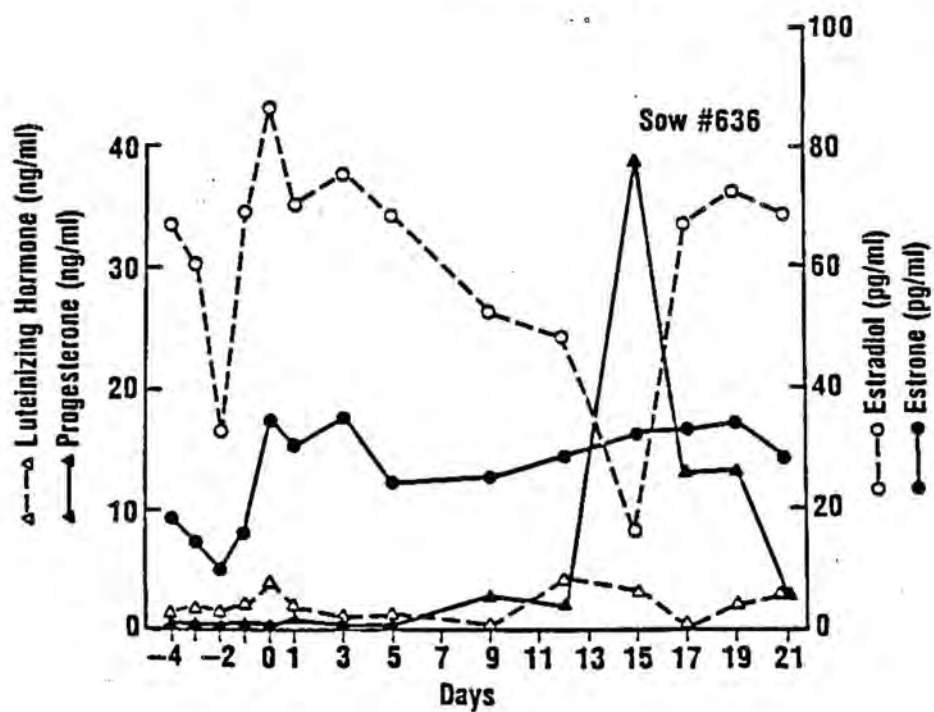
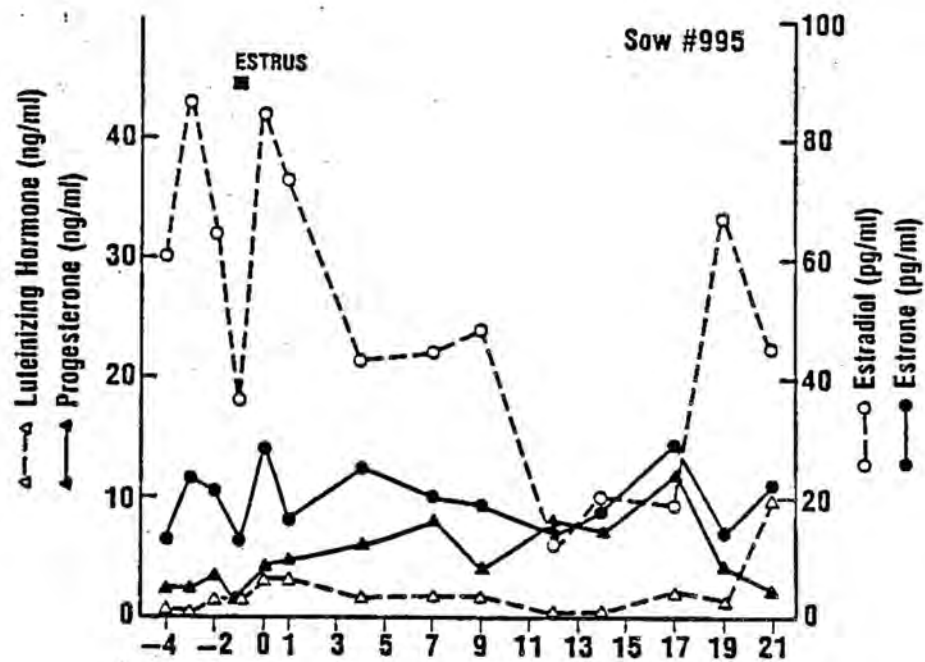
Appendix II. Additional Reagents and Buffers for Luteinizing Hormone  
RIA

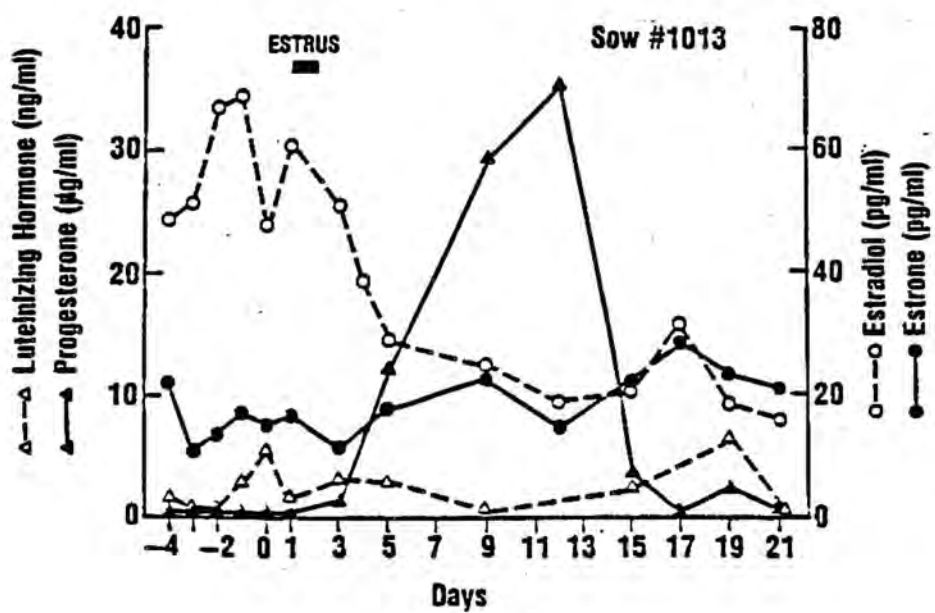
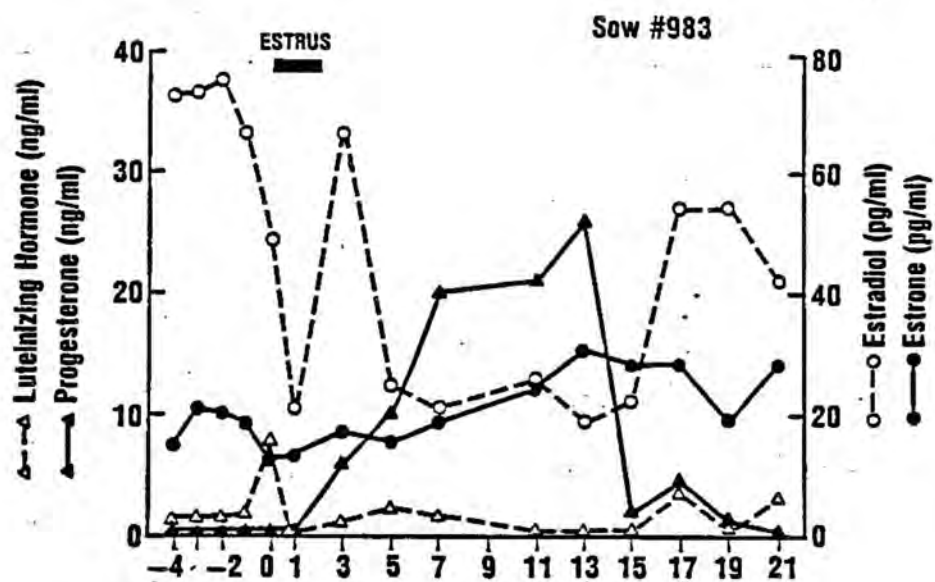
1. 0.05M EDTA-PBS (ethylenediaminetetraacetic acid-PBS)
  - a. Weigh 18.6125 g disodium EDTA.
  - b. Dissolve EDTA in 80 ml of PBS.
  - c. Adjust pH to 7.0 and store at 4°C.
2. NRS Solution (1:400 normal rabbit serum)
  - a. Thaw NRS stock in warm water.
  - b. Take 0.125 ml of NRS stock and dilute with 49.875 ml of 0.05M EDTA-PBS.

## Appendix III. Reagents and Buffers for Steroid Hormone Receptor Assay

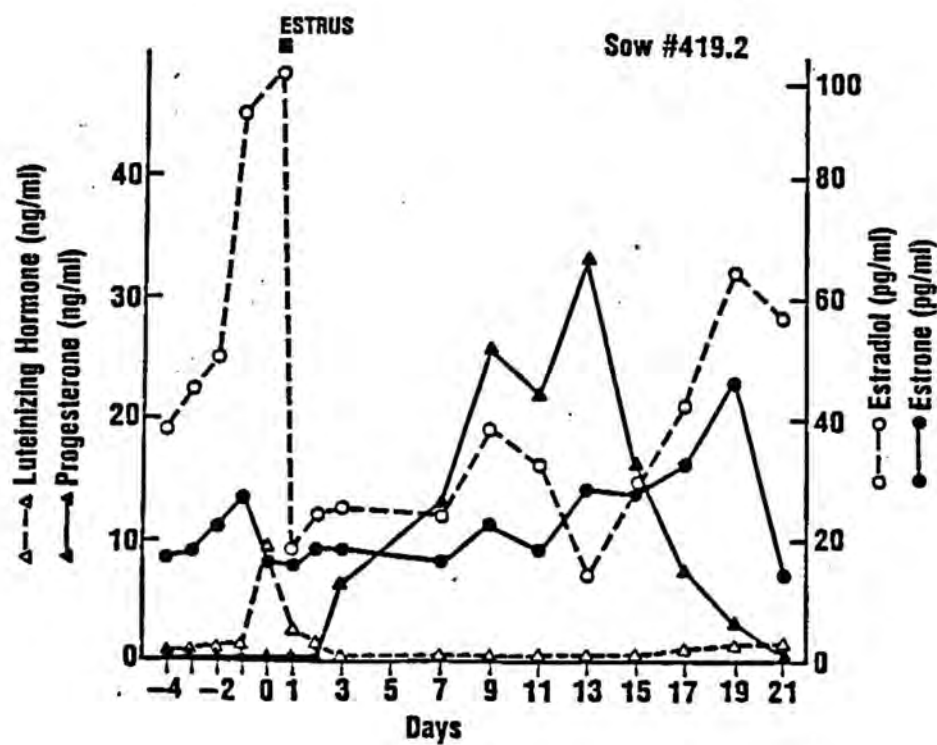
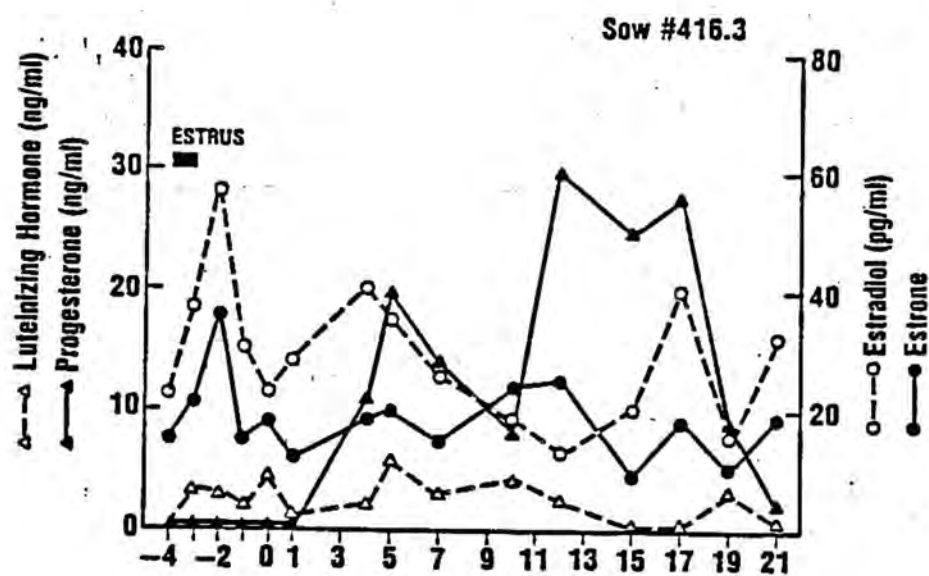
1. TE Buffer (Tris-EDTA, 10mM Tris, 1mM EDTA)
  - a. Weigh 3.904 g Tris-HCL.
  - b. Weigh 1.84 g Tris (basic).
  - c. Weigh 1.488 g EDTA.
  - d. Dilute to 4 L with distilled water.
  - e. Adjust pH to 7.4 and store at 4°C.
2. TED Buffer (Tris-EDTA-DTT, 10 mM Tris, 1mM EDTA, 1mM dithiothreitol)
  - a. Weigh 0.1543 g DTT.
  - b. Dilute with 1 L TE buffer.
  - c. Adjust pH to 7.4 and store at 4°C.
3. 0.4M KCl Solution
  - a. Weigh 29.82 g KCl.
  - b. Dilute to 1 L with TE buffer.
  - c. Adjust pH to 7.4 and store at 4°C.
4. Dextran-Charcoal Suspension (0.25% charcoal, 0.025% dextran)
  - a. Add 2.5 g charcoal to storage bottle containing magnetic stirring bar.
  - b. Weigh 0.25 g T-70 dextran and add 1 L of TE buffer, while stirring.
  - c. Add to storage bottle containing charcoal and mix thoroughly.
  - d. Adjust pH to 7.4 and store at 4°C.

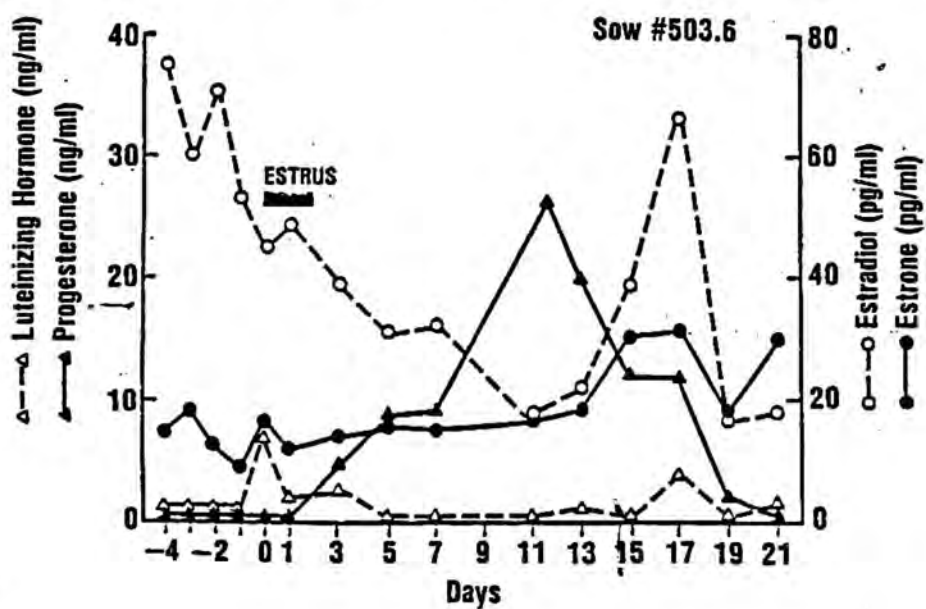
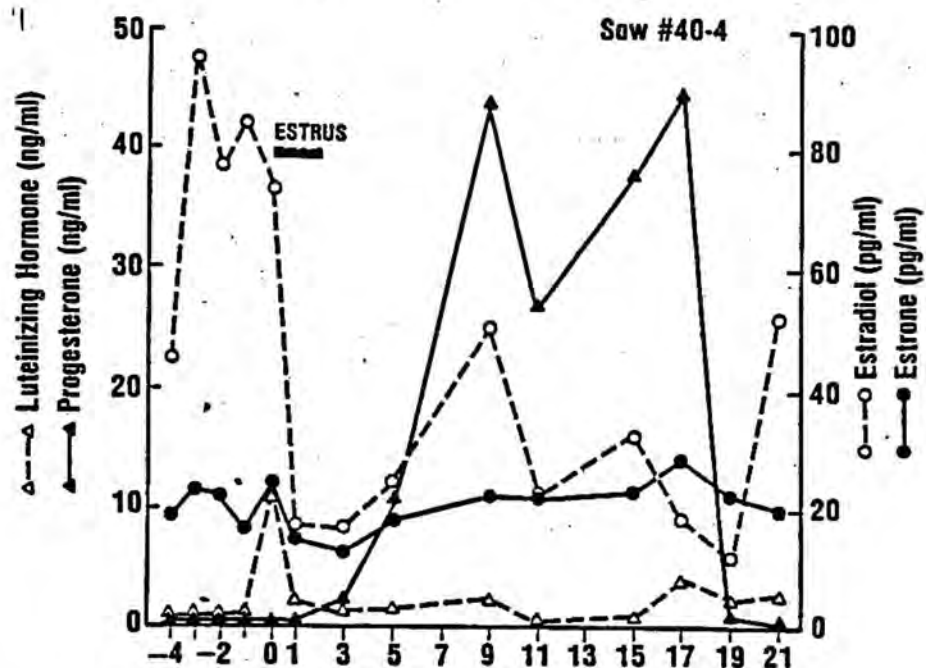
Appendix IV. Serum concentrations of estrone, estradiol, luteinizing hormone and progesterone in individual CEH sows during the synchronized estrous cycle.

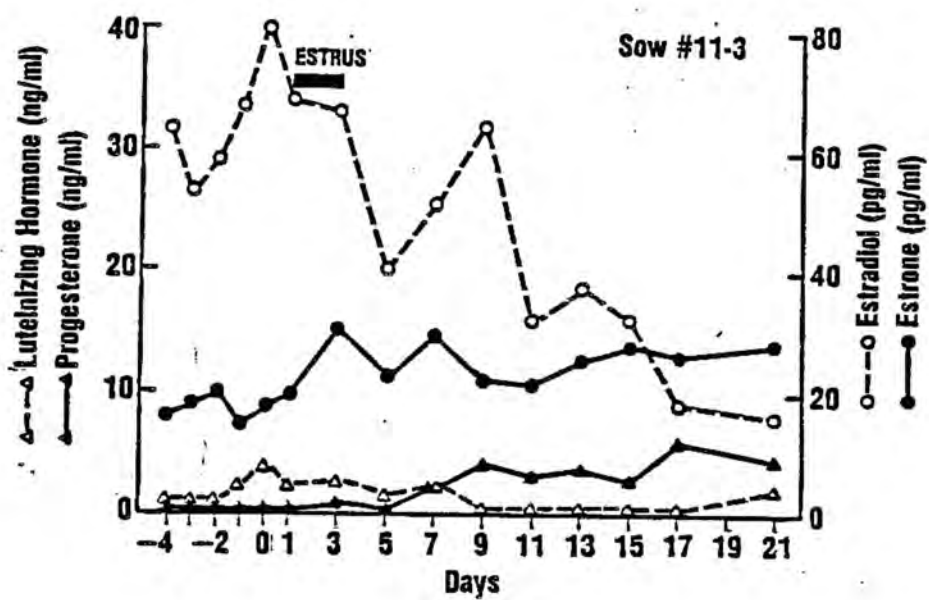




Appendix V. Serum concentrations of estrone, estradiol, luteinizing hormone and progesterone in individual non-CEH sows, during the synchronized estrous cycle.







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